THE BIOCHEMICAL JOURNAL

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THE BIOCHEMICAL JOURNAL

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The Coupled Oxidation of Pyruvate with Glutathione and Cysteine

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Istituto di Patologia Generale, C.N.R. Centro di Fisiopatologia, Rome

(*Received* 19 *April* 1950)

Among the numerous reactions that pyruvate undergoes in biological systems its oxidation to acetic acid and carbon dioxide is at present little understood. A pyruvate dehydrogenase has been reported in animal tissues, but has not been isolated. As regards oxidative decarboxylation we know that acetate is formed from pyruvate by a number of tissues and bacteria (Lipmann, 1937; Barron & Lyman, 1939; Long & Peters, 1939), and that acetate represents an important stage in the biological degradation of pyruvate (Bartlett & Barron, 1947; Kalnitsky & Barron, 1947).

Krebs & Johnson (1937) postulated a dismutation in which one molecule is oxidatively decarboxylated and the other reduced, giving rise to the formation of lactic acid, acetic acid and carbon dioxide. Banga, Ochoa & Peters (1939) found that brain dispersions can oxidize pyruvate provided that cocarboxylase, a C_4 dicarboxylic acid, inorganic phosphate, adenylic acid and magnesium or manganese ions are present. Under these conditions, as Long (1938) showed, 20% of the pyruvate is recoverable as acetic acid. Weil-Malherbe (1940) and Martius (1943) suggested that a reaction between the pyruvic acid and the amino group of the cocarboxylase is necessary both for oxidative decarboxylation and for anaerobic fission. Lipmann (1937, 1939, 1941), working with bacteria, suggested that the pyruvic dehydrogenase oxidizes a pyruvate-phosphate complex formed in the presence of inorganic phosphate, producing carbon dioxide and an energy-rich acetyl phosphate; in the presence of adenosinediphosphate the latter then forms adenosinetriphosphate and acetic acid.

Cavallini (1946) reported an oxidation of pyruvate to acetate during the oxidation of ascorbic acid with a suitable catalyst. This 'induced oxidation' of pyruvate by ascorbic acid may be of biological importance, since according to Cavallini & Bonetti (1946) ascorbic acid is involved in the degradation of pyruvate *in vivo*.

This paper describes experiments on the oxidation of pyruvate with glutatione and cysteine which may be of significance in connexion with the obscure problem of pyruvate breakdown *in vivo*.

EXPERIMENTAL

Materials

Glutathione and *cysteine* (Hoffman La Roche) were used without further purification.

Sodium pyruvate was prepared according to Robertson (1942).

Cytochrome c was prepared according to Keilin & Hartree (1937).

Cytochrome oxidase was prepared from calf heart muscle by the method of Stotz & Hastings (1937). The extract was reprecipitated with acetate buffer (pH 4·5) and resuspended in the original volume of 0·067 M-Na_2HPO_4.

Methods

O_2 uptake and CO_2 evolution were measured by the Warburg technique; conical vessels with single or double side arms were used throughout.

CO_2 evolution was determined by the use of paired vessels containing the same reactants, except that in one of the flasks the NaOH in the centre well was replaced by water. The CO_2 output was then given by the relation

$$x_{CO_2} = \left\{h'' - \frac{h' k'_{O_2}}{k''_{O_2}}\right\} k''_{CO_2},$$

where h' and h'' represent the change in pressure at a given time in the flasks with and without NaOH; k'_{O_2} is the manometric constant for O_2 for the first flask, and k''_{O_2} and k''_{CO_2} the constants for O_2 and CO_2 of the second manometer. Bound CO_2 was estimated by tipping in tartaric acid from a side arm into those flasks which did not contain NaOH in the centre well.

Unless otherwise stated, the main compartment of the flasks contained 1 ml. of 0·067 M-phosphate buffer, pH 7·4; 0·1 ml. of 0·1 N-NaOH (for the neutralization of the cysteine hydrochloride and glutathione solutions); 0·0002 mmol. $CuCl_2$ in 0·3 ml. of water, or 1·72 mg. cytochrome c (0·0001 mmol.) in 0·1 ml. of water + 0·2 ml. cytochrome oxidase preparation; 0·02 mmol. sodium pyruvate in 0·2 ml. of water; water to a total volume of 3 ml. Glutathione or cysteine, 0·02 mmol. in 0·3 ml. of water, was placed in the side arm, while the second side arm (when used) contained about 0·1 g. crystalline tartaric acid. The flasks contained air and the measurements made at 38°; pH values were determined electrometrically.

RESULTS

When glutathione is oxidized either with $CuCl_2$ or with cytochrome *c* and cytochrome oxidase, an uptake of O_2 occurs corresponding to the reaction $2GSH + O \rightarrow GSSG + H_2O$. Under the conditions used in the present work the observed O_2 uptake is very close to the theoretical value of 112 μl./0·02 mmol. glutathione.

If, however, this reaction is allowed to proceed in the presence of sodium pyruvate the O_2 consumption is increased and CO_2 is evolved in excess of the small amount produced during the oxidation of glutathione

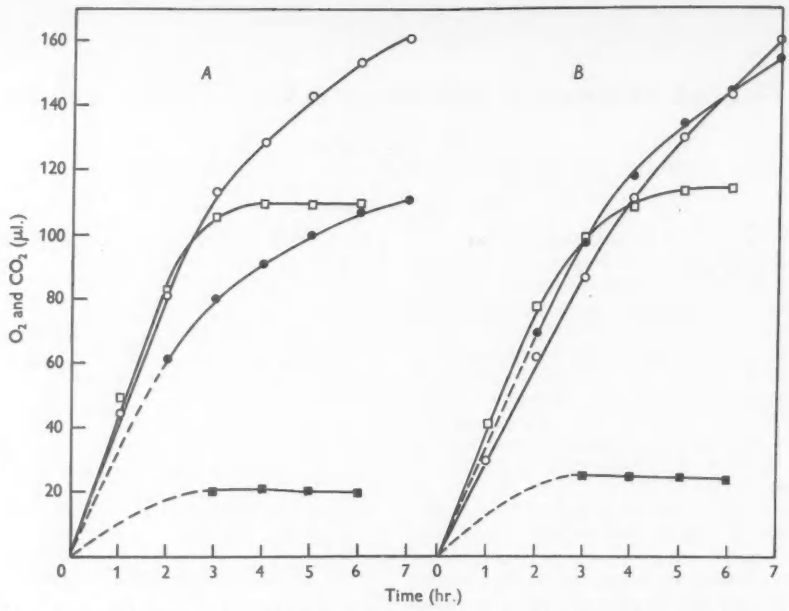


Fig. 1. Oxidation of glutathione. □, O_2 uptake; ■, CO_2 output during the oxidation of 0·02 mmol. glutathione; ○, O_2 uptake; ●, CO_2 output during the oxidation of the same amount of glutathione in the presence of 0·02 mmol. sodium pyruvate. *A*, with 0·0002 mmol. $CuCl_2$ as catalyst; *B*, with 0·0001 mmol. cytochrome *c* + cytochrome oxidase as catalyst.

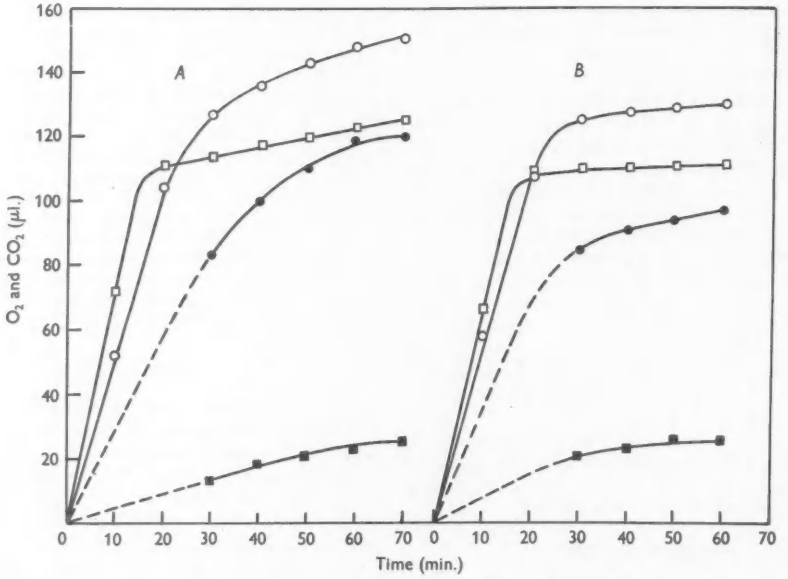


Fig. 2. Oxidation of cysteine. □, O_2 uptake; ■, CO_2 output during the oxidation of 0·02 mmol. cysteine. ○, O_2 uptake; ●, CO_2 output during the oxidation of the same amount of cysteine in the presence of 0·02 mmol. sodium pyruvate. *A*, with 0·0002 mmol. $CuCl_2$ as catalyst. *B*, with 0·0001 mmol. cytochrome *c* + cytochrome oxidase as catalyst.

alone (Fig. 1). (As shown in Figs. 1 and 2 a small CO_2 evolution occurs during the oxidation of both glutathione and cysteine. The origin of this CO_2 is not understood.) Since the method of estimating CO_2 output is not accurate in the initial stages of the reaction, the experimental curve for CO_2 is drawn partly as an arbitrary broken line in both Figs. 1 and 2. Fig. 2 shows that the same result is obtained when cysteine is substituted for glutathione.

In the absence of glutathione or cysteine no oxidation of pyruvate was observed under these conditions; furthermore, oxidized glutathione and cystine are without effect. No oxidation occurs when the cupric chloride or cytochrome *c* and cytochrome oxidase are absent, or when potassium cyanide is present.

conditions used here an oxidative decarboxylation to acetic acid and CO_2 occurs.

In the experiments described in Table 2 cysteine was used instead of glutathione, and under these conditions the CO_2/O_2 ratio is different. This difference might be explained by assuming either a different mode of oxidation of the pyruvate or by a partial removal of cysteine by pyruvate in a form which is not catalytically oxidizable by O_2.

Schubert (1935, 1936, 1939) has studied the compounds formed by interaction of thiols with aldehydes, pyruvic acid and sugars. He found that in the case of glutathione, thioglycollic acid and its derivatives, addition compounds (I) are formed. In the case of cysteine and thiourea, in which an amino group is adjacent to the thiol group, a condensation

Table 1. *Effect of increasing concentration of pyruvate on the oxidation of pyruvate by glutathione*

(Glutathione (0·02 mmol.) dissolved in 0·3 ml. water in side arm. In the main flask: 1 ml. phosphate buffer; 0·4 ml. 0·1N-NaOH; $CuCl_2$, 0·0002 mmol. in 0·3 ml.; sodium pyruvate in different quantities. Centre well: 0·2 ml. 10% (w/v) NaOH, or 0·2 ml. water (see Experimental section). Total vol. 3 ml. Temp. 38°. Gas space filled with air.)

Pyruvate added (mmol.)	pH	Time of oxidation (hr.)	O_2 (μl.)	CO_2 (μl.)	Extra O_2 (μl.)	Extra CO_2 (μl.)	Extra CO_2/O_2
0	7·70	3·5	112·0	28·4	—	—	—
0·005	8·20	4	123·0	48·3	11·0	19·9	1·81
0·01	7·70	4	145·5	100·8	33·5	72·4	2·16
0·1	8·05	5	183·0	159·0	71·0	130·6	1·84

From these results is would appear that a secondary oxidation of the added pyruvate takes place during the oxidation of either glutathione or cysteine.

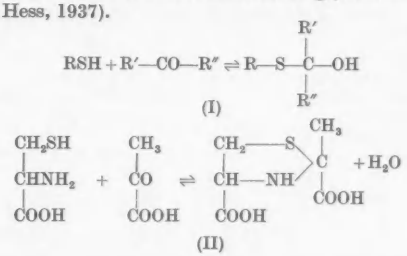
Table 2. *Effect of increasing concentration of pyruvate on the oxidation of pyruvate by cysteine*

(Cysteine (0·02 mmol.) dissolved in 0·3 ml. water in side arm. In the main flask: 1 ml. phosphate buffer; 0·1 ml. 0·1N-NaOH; $CuCl_2$, 0·0002 mmol. in 0·3 ml.; sodium pyruvate in different quantities. Centre well: 0·2 ml. 10% (w/v) NaOH, or 0·2 ml. water (see Experimental section). Total vol. 3 ml. Final pH 7·3. Time of oxidation 1 hr. Temp. 38°. Gas space filled with air.)

Pyruvate added (mmol.)	O_2 (μl.)	CO_2 (μl.)	Extra O_2 (μl.)	Extra CO_2 (μl.)	Extra CO_2/O_2
0	123·0	23·1	—	—	—
0·005	129·8	78·4	6·8	55·3	8·15
0·01	132·0	91·4	9·0	68·3	7·60
0·02	148·1	119·2	25·1	96·1	3·82
0·1	106·0	178·4	—	153·3	—

Table 1 shows the results of experiments in which the reaction between glutathione and different amounts of pyruvate was allowed to go to completion. The CO_2 production and O_2 uptake in excess of the amounts required for the oxidation of the glutathione alone were assumed to be concerned with the oxidation of the added pyruvate. The CO_2/O_2 ratio is close to 2, over the range of pyruvate used. This result suggests, therefore, that under the

product (II) is formed by the elimination of water and the closure of the thiazolidine ring (see Sullivan & Hess, 1937).



This difference in the behaviour of the two thiol compounds when reacting with carbonyl groups would explain the different results in the oxidation of pyruvate. Since Schubert showed that both addition and condensation products are, in solution, in equilibrium with their components, an increase in pyruvate would displace the equilibrium towards the right in reactions (I) and (II). With glutathione, increase in pyruvate leads to a greater concentration of the addition compound, but the oxidation of pyruvate is more complete and the CO_2/O_2 ratio remains constant, suggesting that formation of an addition complex represents a stage in the coupled oxidation of pyruvate by glutathione. With cysteine, increase in pyruvate favours not only the formation of the addition compound (which we may presume

to occur as an intermediate product), but also the condensation of the two substances; the depression of the O_2 uptake observed when the concentration of pyruvate is increased, and the variable CO_2/O_2 ratios could be explained by the partial exclusion of cysteine from oxidation owing to formation of the condensation product. Since the latter, like cysteine, reduces iodine (Schubert, 1936) it is possible to estimate it in solution at the end of the catalytic oxidation.

The data in Table 3 support this explanation. Pyruvate and cysteine were incubated in the side arm for different periods in the absence of the catalyst in order to facilitate the formation of the condensation product; the contents of the side arm were then tipped into the main flask containing the catalyst and the oxidation allowed to proceed to completion. At the end of the experiment the contents of the vessels were deproteinized by the addition of 2 ml. of 10% (w/v) trichloroacetic acid and centrifuged. The supernatant was neutralized with solid Na_2CO_3 and titrated with I_2, and the results were compared with the amount of I_2 utilized by 0·02 mmol. cysteine treated in the same way. It was found that as the preliminary incubation period was increased the O_2 uptake decreased and a greater quantity of cysteine was estimated iodometrically.

Table 3. *Effect of the incubation of cysteine with pyruvate for different times before contact with the catalyst*

(In the main flask: 1 ml. phosphate buffer; 0·1 ml. 0·1 N-NaOH; cytochrome *c*, 0·0001 mmol. (1·72 mg.) in 0·1 ml.; 0·2 ml. cytochrome oxidase preparation. Centre well: 0·2 ml. 10% (w/v) NaOH, or 0·2 ml. water (see Experimental section). Total vol. 3 ml. Final pH 7·3. Temp. 38°. Gas space filled with air. Cysteine, 0·02 mmol. in 0·3 ml. water, and pyruvate, 0·02 mmol. in 0·2 ml. water, were placed together in the side arm just before immersion of the vessels in the water bath. After the desired time, the contents of the side arm were tipped into the main space and the oxidation left to proceed completely (1 hr.). For the recovery of non-oxidized cysteine and calculation see text, p. 4.)

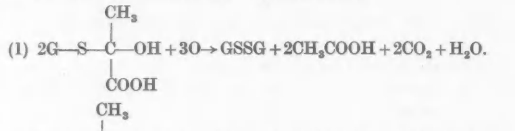
Time of incubation of cysteine with pyruvate before tipping (min.)	O_2 (μl.)	CO_2 (μl.)	Cysteine recovered at the end of oxidation (%)	
			By I_2 titration	Calculated
0	130·0	97·4	10·0	18·4
15	98·0	80·2	48·4	47·0
30	80·0	77·4	51·2	54·0
60	58·8	61·6	74·0	74·8

Under the same conditions cysteine alone consumed 112 μl. O_2.

If the oxidation of pyruvate proceeds as suggested above, it is possible to estimate from the amount of CO_2 evolved the amount of cysteine excluded from oxidation. Since the amount of O_2 needed for the decarboxylation of the pyruvate is equal to half the CO_2 evolved, the O_2 needed for the oxidation of cysteine is $O_2-\frac{1}{2}CO_2$, while $112-(O_2-\frac{1}{2}CO_2)$ represents the cysteine excluded from oxidation in the form of a condensation compound. (The CO_2 evolved during the oxidation of the cysteine alone, 26·6 μl., has been neglected. The slight correction involved does not affect the results.) As is shown in the last two columns of Table 3, this manometric calculation is in reasonable agreement with the iodometric titration; it further shows that with cysteine also the CO_2/O_2 ratio for the coupled oxidation of pyruvate is approximately 2. That the condensation product of cysteine with pyruvic acid is not catalytically oxidized by molecular oxygen has been confirmed by preparing it according to Schubert (1936) and subjecting it to the action of the catalyst; under the same conditions as those described in Table 3 this compound gave only a low rate of oxidation (11·5 μl. O_2/hr.), this low uptake being probably due to a low hydrolysis of the condensation compound to the addition compound.

DISCUSSION

From the above experiments it may be concluded that if pyruvic acid is present in the reaction mixture a secondary oxidation may occur in the course of the oxidation of glutathione or cysteine From the extra carbon dioxide/oxygen ratios obtained in the presence of pyruvic acid it may be argued that this oxidation of pyruvate proceeds through an oxidative decarboxylation to acetate and carbon dioxide. Since glutathione, which is known to form only an addition compound, can perform the coupled oxidation of pyruvate, and since cysteine is not oxidizable when it is forced to form a condensation product, it is suggested that the reaction proceeds by means of the addition, and not the condensation, of pyruvic acid with the thiol group. Either of the following reactions might be postulated:



Free sulphydryl groups are known to be a limiting factor in the metabolism of pyruvate; evidence for this was provided by Peters & Wakelin (1946) using maleate, and Peters, Sinclair & Thompson (1946) using arsenical vesicants.

Aneurin, one of the main factors concerned with the catabolism of pyruvate, was shown by Zima & Williams (1940) and Zima, Ritsert & Moll (1941) to be capable of undergoing reversible oxidation and reduction, $2RSH \rightleftharpoons RSSR$, both the reduced and oxidized forms having equal biological activity. The question arises, therefore, whether the catalytic activity of aneurin is due to oxidation of an addition compound formed between pyruvate and the reduced form of the vitamin, in a manner similar to the coupled oxidation of pyruvate with glutathione described above.

SUMMARY

1. When glutathione or cysteine is oxidized either with copper ions or with the cytochrome *c*-cytochrome oxidase system, a secondary oxidation occurs when pyruvate is added.

2. The ratio of the extra carbon dioxide output to the extra oxygen uptake due to the added pyruvate is approximately 2, suggesting an oxidative decarboxylation to acetic acid and carbon dioxide.

3. It is suggested that this coupled oxidation of pyruvate with glutathione may throw light on the biological oxidation of pyruvate.

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The Effect of Amino-acids on the Growth of *Lactobacillus casei* A.T.C. 7469 in Pteroyl Glutamic Acid-Free Media

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Most media suggested for the estimation of pteroyl glutamic acid (PGA), using *Lactobacillus casei* as a test organism, contain hydrolysed casein plus tryptophan and cystine as a nitrogen source. Snell (1944) found that the growth response of this organism to PGA was increased by supplementing such media with DL-α-alanine (hereafter referred to as alanine). Dolby & Waters (1944) reported that leucine, isoleucine and threonine behaved in a similar manner. Subsequently a number of workers have recommended the inclusion of alanine (1–2 mg./10 ml.) in such media (Tepley & Elvehjem, 1945; Kreuger & Peterson, 1945, and Kodicek & Mistry, 1949).

During experiments on the formulation of a medium for the estimation of PGA with *Lb. casei*, it was found that addition of alanine to a medium containing a commercial brand of 'vitamin-free' casein hydrolysate, resulted in good growth in the absence of added PGA. When alanine was omitted no growth was obtained unless PGA was added. This unexpected behaviour on the part of the test

organism, was attributed to one or more of the following possible causes: (1) The ability of the organism to grow in a medium of a certain amino-acid content rich in alanine, in the absence of PGA. (2) The presence in the casein hydrolysate of a trace of PGA insufficient to produce growth without the addition of alanine, but sufficient to produce growth if alanine was present. (3) The presence of PGA in the inoculum, carried over from the inoculum medium. (4) The use of a culture of *Lb. casei* which had either developed atypical characteristics with regard to its PGA requirement, or which had become contaminated with another glucose fermenting organism with no PGA requirement.

Tests were made to investigate these possibilities and during the course of these L-glutamic acid was also found capable of stimulating the growth of *Lb. casei* in a PGA-free medium.

EXPERIMENTAL

The test organisms used were obtained from two cultures of *Lb. casei* A.T.C. 7469 maintained by different laboratories. One culture had been maintained in yeast-water-glucose-agar medium and the other in liver-tryptone-agar medium.

The inocula were prepared by transferring the organism from the stab culture to 10 ml. of PGA-free medium to which was added 2 μmg. PGA (Stokstad & Hutchings, 1947). After incubating for 20–24 hr. at 37° the cells were centrifuged out and washed with 10 ml. of saline. This process was repeated until the cells had been washed (*a*) four times, (*b*) five times. The cells were finally suspended in saline and the suspension diluted to contain approximately 20 million organisms/ml. One drop of this suspension was added to each tube for the tests. The media used contained as a nitrogen source either (*a*) a commercial acid-hydrolysed casein, stated to be free from vitamins and peptides, which was used in a concentration of 50 mg./10 ml. of medium together with 2 mg. DL-tryptophan and 0·8 mg. of L-cystine or (*b*) the following crystalline amino-acids: L-cystine, DL-tryptophan, DL-leucine, DL-isoleucine, DL-valine, DL-methionine, L-tyrosine, DL-phenylalanine, DL-threonine, L-lysine HCl, L-arginine, DL-serine, DL-norleucine and glycine, 2 mg. of each/10 ml. of medium plus 4 mg. of DL-aspartic acid and DL-histidine. The media differed from each other in some cases in their buffer and vitamin content as shown in Table 1. Growth was measured in terms of acid production after 96 hr. incubation at 37°. Four replicates were made of each test, the results shown in the graphs being the mean values. The linearity of graphical relationships was confirmed statistically.

The tubes of medium prior to inoculation were prepared aseptically and sterilized by heating in an autoclave at 5 lb. pressure for 5 min.

RESULTS

Preliminary tests showed that cells prepared by four or five washes from either culture, produced an identical amount of growth in media containing either of the two types of nitrogen source used without addition of PGA but with additional alanine. It was therefore concluded that the growth, obtained in the absence of added PGA, was due neither to the use of a culture which had acquired an atypical PGA requirement as a result of continued subculture in one particular type of medium, nor to the presence of PGA in the inoculum.

No organisms other than *Lb. casei* were found in those tubes which showed growth in the absence of added PGA. In the case of tests made in the amino-acid medium, the organisms growing in the tubes containing the lowest concentrations of alanine and L-glutamic acid used, were found, when stained, to differ in appearance from the normal organism. The cells had a clubbed appearance and showed no palisade arrangement. On subculture in glucose-

Table 1. *Composition of media used in tests*

(Nitrogen source as indicated in the text. pH 6·8.)

Component	Quantity in 10 ml. of medium				
	1	2	3	4	5
Sodium acetate (anhydrous) (mg.)	36	60	—	—	—
Potassium acetate (mg.)	—	—	36	300	300
Glucose monohydrate (mg.)	200	200	200	300	300
Uracil, guanine, adenine and xanthine—of each (mg.)	0·1	0·1	0·1	0·1	0·1
Aneurin, nicotinic acid, riboflavin and calcium D-pantothenate—of each (μg.)	5	5	5	10	10
Pyridoxine hydrochloride (μg.)	10	10	10	25	25
p-Aminobenzoic acid (μg.)	1	1	1	2	2
Biotin (μmg.)	4	4	4	4	4
Salt solution 'A' (ml.)*	0·05	0·05	0·05	0·05	0·05
Salt solution 'B' (ml.)*	0·05	0·05	0·05	0·05	0·05
Sodium chloride solution 0·2% (w/v) (ml.)	0·05	0·05	0·05	0·05	0·05
Water to	10 ml.	10 ml.	10 ml.	10 ml.	10 ml.

* Salt solution 'A' and 'B', Microbiological Panel of the Society of Public Analysts (1946).

yeast medium, however, they produced cells having the morphological and biochemical characteristics of *Lb. casei* A.T.C. 7469 (Kodicek & Pepper, 1948). Organisms in tubes containing a higher concentration of these amino-acids, showed the normal rod-shaped appearance but no palisade arrangement except when they were subcultured in glucose-yeast medium. Organisms in tubes containing the highest concentrations of these amino-acids used had the normal appearance of the organism and also showed palisade formation.

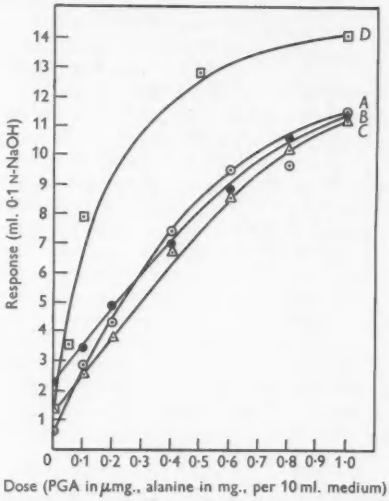

Fig. 1. The effect of PGA and alanine on the growth of *Lb. casei* in pteroyl glutamic acid (PGA)-free media containing a casein hydrolysate nitrogen source. Curve *A*, ⊙, medium no. 1, responses to PGA additions; curve *B*, ●, medium no. 2, responses to PGA additions; curve *C*, △, medium no. 3, responses to PGA additions; curve *D*, ⊡, medium no. 3, responses to alanine additions without PGA.

The liability of old cultures of *Lb. casei* to show pleomorphism and irregular beaded staining, as reported by Kodicek & Pepper (1948), is a characteristic of the Lactobacillus group in general.

Variations in morphology are also reported (Topley & Wilson, 1945) to be produced by growing the organisms in artificial media as distinct from media of natural origin. These observations suggest that the occurrence of changes of morphology in an organism when grown in a certain medium is an indication that that medium is deficient in substance(s) necessary for normal growth.

Tests made in media 1, 2 and 3 (Table 1) containing casein hydrolysate gave the responses, shown in Fig. 1 (curves *A–C*), to additions of PGA. These media gave low blank titres and good agreement between replicates. The relation between log dose of PGA and response was found to be linear in the dose range 0·2–1·0 μmg. PGA/10 ml. The addition of alanine to medium 3 in the absence of PGA resulted in growth, the amount of growth obtained by the addition of 1 mg. alanine/10 ml., exceeding that produced by 1 μmg. of PGA/10 ml. (Fig. 1, curve *D*). A similar effect was obtained by adding alanine to medium 4 (Table 1), having the same amino-acid content as the three previous media, but differing in vitamin content (Fig. 2).

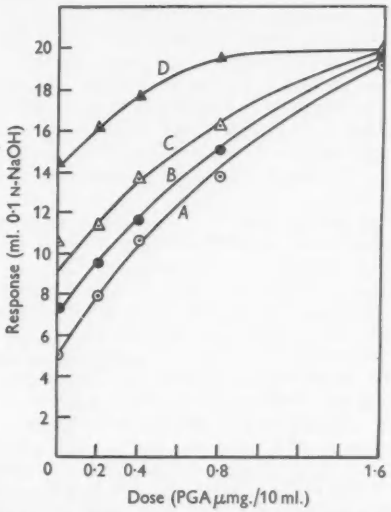

Fig. 2. The effect of alanine on the growth, response of *Lb. casei* to additions of pteroyl glutamic acid (PGA) in a PGA-free medium (no. 4) containing a casein hydrolysate nitrogen source. Curve *A*, ⊙, 0·02 mg. alanine/10 ml. medium; curve *B*, ●, 0·04 mg. alanine/10 ml. medium; curve *C*, △, 0·08 mg. alanine/10 ml. medium; curve *D*, ▲, 0·16 mg. alanine/10 ml. medium.

The relation between log concentration of alanine (log dose) and growth response was also found to be linear, over the range examined, namely 0·02–0·16 mg./10 ml. of medium. Addition of increasing amounts of PGA to medium 4 containing different amounts of alanine, produced equal increases in growth response irrespective of the amount of alanine present over the range 0·02–0·16 mg. alanine/10 ml., though the growth in the blank test (no PGA) increased with increasing alanine content, i.e. the curves relating PGA addition and growth, over the range 0·2–0·8 μmg. PGA/10 ml., were parallel at all the concentrations of alanine examined (Fig. 2). This means that in the assay of PGA with these media the blank increases markedly with increasing alanine content of the medium, and that

the response produced by a mixture of PGA and alanine in this medium is the summation of the effects of each.

In the ranges investigated, the ratio of the doses of alanine and PGA producing equal growth is approximately 250,000 : 1. Samples containing alanine and PGA assayed with this medium for their PGA content would probably not contain enough alanine, relative to the amount of PGA present, to interfere seriously with the assay. It is shown later that tests made with the medium containing amino-acids as a source of nitrogen indicate that a change in the amino-acid content of the medium may specifically increase the response to alanine, so that the relative amounts of PGA and alanine producing the same response are not so widely separated. The apparent potencies of natural materials will, therefore, be liable to vary when assayed for their PGA content with *Lb. casei* using media of different amino-acid content as stated by Snell (1944).

Snell (1944) found that the addition of 2 mg. of alanine/10 ml. of medium increased the growth response to PGA by a maximum of 80%.

Kreuger & Peterson (1945) found that the addition of 1 mg. of alanine/10 ml. of medium increased the growth response to PGA by 10%.

The response obtained in the present experiments to the traces of PGA possibly present in the casein hydrolysate (represented by the blank titre in the tests) was 1·2 ml. 0·1N-sodium hydroxide; the response to this same amount of PGA obtained when 1 mg. of alanine was added to the medium was 14 ml. 0·1N-sodium hydroxide. This is approximately ten times greater than that obtained by Snell (1944), although the concentration of alanine was half that used in the tests he described.

The effect of addition of alanine in the present experiments is so much greater than the effect quoted by Snell that it is considered that the growth produced by alanine in these experiments cannot be due to traces of PGA in the casein hydrolysate. This conclusion was confirmed by the tests made with the amino-acid medium.

Further tests on the effect of alanine on the growth of *Lb. casei* in PGA-free media were made in medium 5 (Table 1) containing a mixture of amino-acids as the source of nitrogen. Tests were also made to determine the extent to which the growth-producing effect of alanine depended on the amount of L-glutamic acid present—this amino-acid being part of the PGA molecule, and therefore possibly connected with the role of PGA in the nutrition of *Lb. casei*.

The results showed that the amount of growth produced by a fixed amount of alanine depended on the amount of L-glutamic acid present in the medium up to a maximum L-glutamic acid content of 5 mg./10 ml. It was also found that in the presence of a fixed amount of L-glutamic acid growth was increased by increasing the quantity of alanine added. No growth was obtained in the absence of

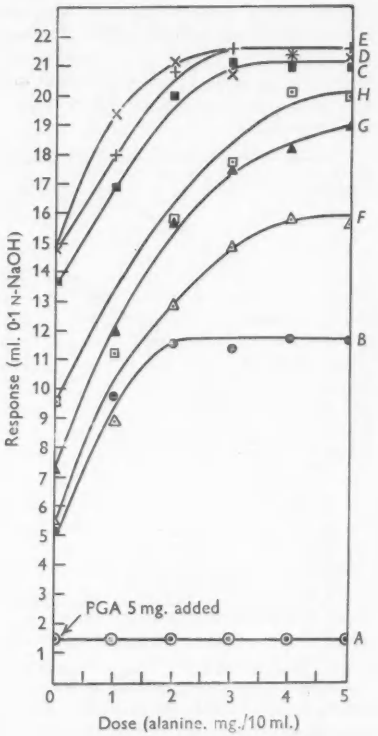

Fig. 3. The effect of alanine and L-glutamic acid on the growth of *Lb. casei* in a pteroyl glutamic acid (PGA)-free medium (No. 5) containing a mixture of crystalline amino-acids as a nitrogen source.

Curve *A*, ⊙, 0 mg. L-glutamic acid/10 ml. medium
Curve *B*, ●, 1·0 „ „ „ „ „
Curve *C*, ■, 2·5 „ „ „ „ „ } Set 1
Curve *D*, +, 5·0 „ „ „ „ „
Curve *E*, ×, 10·0 „ „ „ „ „
Curve *F*, △, 1·5 „ „ „ „ „
Curve *G*, ▲, 2·0 „ „ „ „ „ } Set 2
Curve *H*, ⊡, 2·5 „ „ „ „ „

Curves *A*–*E* and *F*–*H* were obtained in separate experiments at different times.

L-glutamic acid even when as much as 5 mg. of alanine was added to 10 ml. of medium, neither was any growth obtained when 5 mg. of PGA was added to 10 ml. of medium in the absence of L-glutamic acid and alanine. In the media containing 1·5, 2·5

and 5 mg./10 ml. of L-glutamic acid there was a statistically linear relationship between the responses and log. dose additions of alanine.

DISCUSSION

These results confirm the stimulatory effect of additions of alanine on the growth of *Lb. casei* in the presence of PGA in a medium containing hydrolysed casein, and show that in this medium growth occurs in the absence of PGA when alanine is added to the medium. In a medium consisting of a mixture of amino-acids the amount of growth produced by addition of alanine is influenced by the amount of L-glutamic acid in the medium.

It is possible that other amino-acids are involved either in stimulating growth in the presence of alanine, or in retarding growth in its absence. Hutchings & Peterson (1943) report that alanine is able to counteract the inhibitory effect on growth of histidine and isoleucine, producing a combination which has a marked stimulatory effect. Camien & Dunn (1950) have recently shown that glycine and serine also have an effect on the use of alanine by Lactobacilli. The effect of added alanine will, therefore, be also influenced by the quantity of these amino-acids in the medium.

From the tests made it appears that the amino-acid content of PGA-free media could be adjusted to give the optimum response to additions of alanine or L-glutamic acid and it might be possible to use *Lb. casei* in such media for the assay of these amino-acids.

Growth of *Lb. casei* in the absence of PGA has also been reported in the presence of other nitrogenous organic compounds. Stokstad (1941) observed that thymine would support half maximum growth of *Lb. casei* in PGA-free media containing adenine in concentrations of 3 μg./10 ml. medium. It was also found that guanine and other purines had the same effect. It is possible that in the cases where growth was restricted to half maximum, limitation of growth was caused by an insufficiency in the medium of the other components necessary for growth, possibly L-glutamic acid or alanine.

The reason for the failure of addition of alanine to stimulate the growth of *Lb. casei* in the absence of PGA in the media used by those workers recommending this addition, may have been that the casein hydrolysate used in their media had a different amino-acid content from the one used in the present tests. Alanine added to some PGA-free media may be used in neutralizing certain growth-restricting factors, for example histidine and isoleucine.

It follows, therefore, that it is not sufficient to add fixed quantities of amino-acids to media containing protein hydrolysates; each medium has to be considered separately and the optimum amounts of supplementary amino-acids determined.

The amino-acid composition of a casein hydrolysate depends on the conditions of hydrolysis and the method used for removing the vitamins. Thus, Lugg (1938) and Boyd & Logan (1942) have reported that when casein is hydrolysed by acid the threonine, tyrosine, cystine and serine are destroyed to varying extents depending on conditions of hydrolysis. Bailey, Chibnall, Rees & Williams (1943) have also reported that the conditions of hydrolysis will also determine the degree to which the amino-acids have been racemized. This racemization will also affect the growth response since with some amino-acids only the L-forms are active in promoting growth (Kuiken, Norman, Lyman, Hale & Blotter, 1943). Treatment of the hydrolysate with adsorbing agents such as activated charcoal and fuller's earth to remove the vitamins will also cause a certain amount of selective adsorption of the amino-acids, with consequent alteration in their final proportions. Even with media prepared from the crystalline amino-acids slight variations in the final amino-acid content may result from differences in the method of autoclaving the final media and from differences in the purity of amino-acids obtained from different commerical sources.

SUMMARY

1. The titres of the blanks in media used for the microbiological estimation of pteroyl glutamic acid (PGA) with *Lactobacillus casei* depend on the amino-acid content of the media and in particular on the amount of L-glutamic acid and DL-α-alanine present.

2. Unrestricted growth of *Lb. casei* was obtained in PGA-free media containing certain combinations of L-glutamic acid and DL-α-alanine indicating that PGA could not be considered an essential growth factor for *Lb. casei* under those conditions.

3. The amount of DL-α-alanine needed in media used for the estimation of PGA must be determined by experiment for each medium with a different amino-acid content, such as may arise, for example, from the use of casein hydrolysates of different origins.

4. The possibility of using PGA-free media of a defined amino-acid content for the assay of DL-α-alanine and L-glutamic acid is suggested.

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The Action of Some Water-soluble Poly-α-amino-acids on Blood Clotting

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The action of naturally occurring acidic and basic substances of high molecular weight, such as heparin and protamine, on blood clotting is well known (for review see Chargaff, 1945) and the study of the anti-coagulant effect of these substances has added much to the understanding of the blood-clotting mechanism. The recent synthesis of water-soluble poly-α-amino-acids (for review see Katchalski, 1950) makes possible the study of the action of polymers of well defined structure on blood coagulation. As the poly-α-amino-acids represent materials of relatively high molecular weight, composed of amino-acid residues and linked by peptide bonds, the study of their action on blood coagulation may serve to elucidate some aspects of the mechanism of action of simple proteins, such as protamine, on blood coagulation.

The water-soluble poly-α-amino-acids may be classified according to their electrochemical properties into neutral, basic and acidic poly-amides. In the present study poly-DL-alanine (Astbury, Dalgliesh, Darmon & Sutherland, 1948), the only known neutral water-soluble poly-α-amino-acid, was chosen as representative of the neutral type; the basic poly-α-amino-acids are represented by synthetic poly-lysine (Katchalski, Grossfeld & Frankel, 1948) and poly-ornithine (Katchalski & Spitnik, 1949) and the acidic poly-α-amino-acids by the naturally occurring poly-D-glutamic acid (Hanby & Rydon, 1946) and the synthetic poly-aspartic acid (Frankel & Berger, 1949).

It has been found that neutral and acidic poly-amino-acids do not retard clotting of human blood at a concentration of about 100 μg./ml. blood. Basic poly-amino-acids, on the other hand, cause a marked prolongation of clotting time at the same concentration. The clot-decelerating effect of the basic poly-amino-acids, poly-lysine and poly-ornithine, can be neutralized by the acidic poly-amino-acids as well as by heparin. The basic poly-amino acids act as antiheparins *in vitro*, similarly to protamine. Experiments with poly-L-lysine and poly-DL-lysine showed that variation of the steric configuration does not change significantly their effect on blood clotting. Lysine, ornithine and aspartic acid monomers were found to have no influence on blood clotting. The mode of action of the basic poly-amino-acids on blood clotting was investigated. The similarity of their action to that of protamine will be pointed out.

EXPERIMENTAL

Materials

Poly-L-lysine hydrochloride. This was prepared from L-lysine hydrochloride according to Katchalski *et al.* (1948). Average degree of polymerization (n) = 35 lysine units. In order to determine whether the poly-lysine synthesized contained L-lysine units, the specific rotations of poly-lysine hydrochloride and of the lysine monomer formed on hydrolysis with HCl, were determined. Poly-lysine hydrochloride showed in an aqueous solution (1·79 g./100 ml.) a strong laevorotation $[\alpha]_D^{20°} - 65·4°$, whilst the lysine dihydrochloride monomer, derived from the polymer, showed the usual dextrorotation $[\alpha]_D^{20°} + 15·7°$ (determined at a concentration of 1·26 g. lysine/100 ml. aqueous solution containing 10 equiv. of HCl). Since the specific rotation of the lysine formed on hydrolysis of poly-lysine is normal, it is considered that the poly-lysine prepared from L-lysine is built of L-lysine units. The marked negative rotation of the polymer recalls the negative rotation of many natural proteins.

Poly-DL-lysine hydrochloride. This was prepared from DL-lysine hydrochloride (Eck & Marvel, 1934, 1943), in the same way as the optically active poly-L-lysine. Average chain length (n) = 30 lysine units.

Poly-DL-ornithine hydriodide. This was prepared from DL-ornithine hydrobromide according to Katchalski & Spitnik (1949). The polymer synthesized contained an average of 26 ornithine residues.

Poly-D-glutamic acid. The poly-D-glutamic acid prepared from *Bacillus anthracis* was kindly supplied to us by Dr H. N. Rydon (cf. Hanby & Rydon, 1946).

Poly-aspartic acid. This was prepared from β-benzyl ester of *N*-carboxy-L-aspartic acid anhydride according to Frankel & Berger (1949). The polymer synthesized contained on the average 150 aspartic acid residues.

Poly-DL-alanine. This was prepared from *N*-carboxy-DL-alanine anhydride according to Astbury *et al.* (1948).

Protamine sulphate. This was prepared from ripe carp testicles according to Schmiedeberg (1899).

Heparin. A Lederle heparin solution containing 10 mg. Na salt of heparin/ml. was used. The solution had an anticoagulant potency of 1100 international units/ml.

Thrombin. A refined and dried preparation of bovine thrombin (Upjohn Co.) containing 1000 units/ampoule was used.

Bacto-thromboplastin. A dry preparation from rabbit brain (Difco Laboratories Inc.) was used. A saline suspension was prepared according to Quick (1942).

Blood. Human blood was used throughout.

Methods

Clotting time of venous human blood. This was measured on 2 ml. samples in glass test tubes at 37° by the method of Lee & White (1913) as modified by Pohl & Taylor (1937). Normal values are 5–11 min.

Clotting time of human blood treated with poly-α-amino-acids. To each of a series of test tubes containing 0·2 ml. of the poly-amino-acid solutions of various concentrations, 1·8 ml. venous blood were added immediately after venepuncture and the clotting time determined.

Plasma prothrombin time, one-stage procedure according to Quick (1942). Freshly prepared Difco rabbit-brain thromboplastin extract or human-brain extract prepared and preserved according to Aggeler, Howard, Lucia, Clark & Astaff (1946) was used. In this procedure clotting was induced in a mixture of oxalated plasma and thromboplastin by addition of $CaCl_2$. The prothrombin time is the time from the addition of $CaCl_2$ to the moment of clotting. The prothrombin time is influenced not only by variations in prothrombin content, but also by variations in the concentrations of accelerators of prothrombin conversion, antithrombin and fibrinogen (Alexander, de Vries & Goldstein, 1949).

Plasma and serum prothrombin content by the two-stage method of Sternberger (1947) *as applied to serum by Herz, de Vries & Heiman-Hollander* (1950). Plasma was defibrinated by addition of thrombin; the added thrombin was inactivated by incubation. In the first stage of this two-stage procedure prothrombin was completely converted to thrombin by the addition of thromboplastin and $CaCl_2$, while antithrombin activity was blocked with ethanol. In the second stage the thrombin formed was titrated with a fibrinogen substrate. With this method actual prothrombin content was measured and the effect of variations in prothrombin conversion accelerators, antithrombin and fibrinogen, was largely eliminated.

Prothrombin consumption. Prothrombin conversion in samples of serum obtained at various intervals after clotting was stopped by addition of 1 vol. of 0·1 M-sodium oxalate to 9 vol. of serum (de Vries, Alexander & Goldstein, 1949). The serum was incubated for 30 min. to inactivate the thrombin. The difference between the original plasma prothrombin and the serum prothrombin, as a measure of the thrombin formed, provided that all prothrombin consumed is converted to thrombin. In some experiments in which blood clotting was retarded by the addition of a poly-amino-acid, prothrombin consumption was determined in the plasma samples of the unclotted blood.

Antithrombic acitivity of serum. Bovine thrombin was added to the serum investigated and the mixture incubated at 37°. The amount of thrombin left in the mixture after increasing periods of incubation was measured by titration of a sample of the mixture on fibrinogen substrate ($BaSO_4$-treated normal oxalated plasma).

RESULTS

Anticoagulant action of basic poly-α-amino-acids on blood

Effect of water-soluble poly-α-amino-acids on clotting time of blood. Freshly drawn blood (1·8 ml.) was added to 0·2 ml. of aqueous poly-α-amino-acid solution; the clotting time was determined at 37°. The final concentrations of the preparations per ml. of blood mixture and the corresponding clotting times are recorded in Table 1. The effect of some amino-acids and of protamine sulphate on blood clotting is also shown.

Table 1 shows that the basic poly-amino-acids, poly-L-lysine, poly-DL-lysine and poly-DL-ornithine, retard blood coagulation markedly in the concentration used. Their clot-retarding effect is more pronounced than that of protamine. The acidic poly-amino-acids, poly-aspartic acid and poly-D-glutamic acid, as well as the neutral poly-DL-alanine, do not

retard blood clotting in the same range of concentrations. No effect on blood coagulation was observed with the amino-acid monomers tested.

Table 1. *Effect of water-soluble poly-α-amino-acids on clotting time of blood*

Preparation added	Final concentration in blood mixture (μg./ml.)	Clotting time (min.)
Poly-L-lysine hydrochloride	133	>90
Poly-DL-lysine hydrochloride	130	>90
Poly-DL-ornithine hydriodide	134	>90
Poly-aspartic acid	120	6
Poly-D-glutamic acid	125	7
Poly-DL-alanine	120	9·5
Protamine sulphate	134	25
L-Lysine monohydrochloride	128	8·5
DL-Lysine monohydrochloride	130	9·5
DL-Ornithine monohydrochloride	120	7
L-Aspartic acid	120	7
Control*	—	5–11

* In the control experiment 1·8 ml. blood were added to 0·2 ml. 0·9% (w/v) NaCl. The experiments reported in this table were performed on different human bloods whose range of clotting times is given.

Influence of varying concentrations of basic poly-α-amino-acids on clotting time of blood. In several experiments it was observed that clotting time increased with increasing concentrations of the basic poly-amino-acids. In order to compare the activities of the different basic poly-amino-acids, concentrations containing the same number of polar amino groups were tested. The concentrations recorded in Table 2, as base mols, indicate the number of mols

Table 2. *The influence of basic poly-α-amino-acids in varying concentrations (base mols/ml. of blood mixture) on clotting of blood*

Concentration (base mols*/ml. × 10^6)	Clotting times (min.) at varying concentrations: Poly-L-lysine hydrochloride	Poly-DL-lysine hydrochloride	Poly-DL-ornithine hydriodide
2·3	15·5	15·5	11·5
4·6	17·5	21·0	16·0
9·2	30·0	35·0	30·0
18·4	50·0	120	120
36·8	113	120	120
73·6	120	120	120

* In the calculation of the number of base mols, present in 1 ml. of blood-poly-amino-acid mixture, 1 base mol of poly-L-lysine hydrochloride or poly-DL-lysine hydrochloride was taken as equivalent to 164·5 g. of the polymeric hydrochloride, while 1 base mol of poly-ornithine hydriodide was taken as equivalent to 242 g. of the hydriodide.

of amino-acid residues per ml. of blood mixture. In the calculation of the number of base mols, the molecular weight of a lysine hydrochloride residue was taken as 164·5 and that of ornithine hydriodide as 242·0.

Table 2 shows that the clotting time of blood is prolonged to approximately the same extent by equal base mol concentrations of poly-L-lysine, poly-DL-lysine and poly-DL-ornithine.

Mode of anticoagulant action of basic poly-α-amino-acids

Inhibition of thrombin formation by poly-L-lysine. Mixtures of 0·5 ml. poly-L-lysine solutions and 4·5 ml. blood were allowed to clot at 37°. The clotted blood samples were kept at 37° for an additional hour and centrifuged for 10 min. at 2000 rev./min.; 1/9 vol. of 0·1 M-sodium oxalate was added to the sera thus obtained and the prothrombin content determined by the two-stage method (cf. Table 3). The validity of the latter procedure in the present case will be the subject of subsequent discussion.

The data (Table 3) show that prolongation of clotting time by poly-L-lysine is associated with elevated prothrombin levels or incomplete thrombin formation.

Table 3. *Elevated prothrombin levels in serum from poly-L-lysine-treated blood*

Final concentration of poly-L-lysine hydrochloride in the blood mixture (μg./ml.)	Clotting time (min.)	Serum prothrombin, determined by the two-stage test (% of normal)
0	11	1
4	24	3
8	26	13
16	32	33
31	36	15
62	125	53
125	150	27

Inhibition of prothrombin conversion. In order to evaluate the amount of prothrombin consumed in blood treated with different polymers, it was desirable to compare the levels of serum prothrombin after incubation of the blood samples for a fixed period of time. Comparative data on the inhibition of prothrombin conversion by the various basic poly-amino-acids, protamine, and the α-amino-acid monomers, L- and DL-lysine, are presented in Table 4. In each of the blood samples prothrombin was determined 70 min. after preparation of blood mixture. The plasmas of the blood mixtures which did not clot within 70 min. were defibrinated by the addition of thrombin before the determination of prothrombin by the two-stage test.

Poly-DL-lysine and poly-DL-ornithine inhibited prothrombin conversion similarly to poly-L-lysine; L- and DL-lysine monomers, on the other hand, did not interfere with thrombin formation. It is of interest that at a concentration of 4 μg./ml. blood (Table 3) poly-L-lysine hardly interfered with prothrombin conversion; at the higher concentra-

Table 4. *Inhibition of prothrombin conversion by various basic poly-α-amino-acids*

Preparation	Final concentration in blood mixture (μg./ml.)	Clotting time (min.)	Prothrombin remaining 70 min. after venepuncture; two-stage test (% of normal)
Poly-L-lysine hydrochloride	133	>70	50
Poly-DL-lysine hydrochloride	130	>70	19
Poly-DL-ornithine hydriodide	134	>70	45
Protamine sulphate	130	25	3
Protamine sulphate	1000	40	47
L-Lysine monohydrochloride	134	9·5	1
DL-Lysine monohydrochloride	128	8·5	1

Table 5. *Antithromboplastic activity of poly-L-lysine*

Exp. no.†	Thrombo-plastin extract (0·05 ml.)	Poly-L-lysine hydrochloride	Plasma (0·05 ml.)	Saline (0·05 ml.)	Saline (0·05 ml.)	Concentration of poly-L-lysine hydrochloride in the final one-stage mixture (μg./ml.)	Prothrombin time (sec.)
1	*	.	+	*	+	0	17·0
2	*	+	+	*	.	43	28·8
3	*	*	+	.	+	43	44·0

† In each experiment component pairs, indicated by asterisks or by crosses, were pre-incubated for 5 min. at 37°, before performance of the one-stage prothrombin procedure; 0·1 ml. of each of the two pre-incubated mixtures were mixed; 0·1 ml. 0·22 M-$CaCl_2$ was added and the clotting time determined (prothrombin time).

tions, however, of 125–130 μg./ml. blood, prothrombin conversion was markedly inhibited. In this respect, poly-lysine resembles protamine which manifests the same effect at relatively higher concentrations (Table 4).

Antithromboplastic action of poly-L-lysine. Incubation of thromboplastin with poly-L-lysine resulted in marked inactivation of the thromboplastic activity as demonstrated in the one-stage procedure for the determination of prothrombin.

Pre-incubation of thromboplastin extract with poly-L-lysine prolonged the one-stage prothrombin time considerably. The pre-incubation of the plasma with poly-L-lysine at the same concentration prolonged prothrombin time to a slighter extent. This points to an antithromboplastic activity of poly-L-lysine.

Sensitivity of fibrinogen to thrombin in blood treated with poly-L-lysine. Blood (1·8 ml.) was added to each of a series of test tubes containing 250 μg. poly-L-lysine hydrochloride in 0·2 ml. saline. Thrombin solutions of varying concentrations were added 5 min. after mixing. The clotting times of the mixtures were recorded and compared with those of normal blood added to thrombin directly (Table 6).

Table 6 shows that fibrinogen of blood, made incoagulable for more than 80 min. by poly-L-lysine, showed normal sensitivity to concentrations of 1–8 units of thrombin per ml. blood mixture. Similarly, it was shown that fibrinogen in oxalated plasma mixed with poly-L-lysine remained normally sensitive to thrombin.

Table 6. *Sensitivity of fibrinogen to thrombin in blood treated with poly-L-lysine*

Final concentration of thrombin in blood mixture (units/ml.)	Clotting times (sec.)	
	Blood treated with poly-L-lysine	Untreated blood
0	5000	540
1	35	40
2	35	40
4	25	35
8	15	15

Absence of effect of poly-L-lysine on the inactivation of thrombin in serum. A serum poly-L-lysine mixture and a serum-saline mixture were incubated with bovine thrombin at 37°. From time to time 0·2 ml. samples of the mixtures were withdrawn and their thrombin content determined by titration on 0·2 ml. plasma.

Table 7 shows that the antithrombic activity of serum is not influenced by poly-L-lysine in a concentration markedly retarding blood coagulation.

Effect of poly-L-lysine on the prothrombin determination by the two-stage tests. Since poly-L-lysine interferes with prothrombin conversion, it was necessary to check the reliability of the two-stage test for prothrombin determination, in the presence of basic poly-amino-acids. Poly-L-lysine was therefore added to defibrinated normal oxalated plasma and the amount of prothrombin determined by the usual two-stage procedure (Table 8).

Table 7. *Inactivation of thrombin by serum in the presence of poly-L-lysine*

Time of incubation of thrombin with serum-saline or with serum poly-L-lysine mixture (min.)	Composition of the solutions: 1·6 ml. thrombin (200 units/ml.), 1·6 ml. serum, 0·16 ml. saline	Composition of the solutions: 1·6 ml. thrombin (200 units/ml.), 1·6 ml. serum, 0·16 ml. poly-L-lysine hydrochloride solution (1250 μg./ml.)
	Clotting times (min.)	
0·25	5	5
5	7	7
11	13·5	12·6
17	21	22·4
23	32	36·5
30	50	53
35	68	68
40	82	81

Table 8. *The disturbing effect of poly-L-lysine on the prothrombin determination by the two-stage test*

(In this experiment 0·2 ml. of saline or of poly-L-lysine solutions were added to 0·6 ml. of defibrinated normal oxalated plasma, and the usual two-stage procedure was applied.)

Poly-L-lysine hydrochloride; concentration in final mixture (μg./ml.)	Prothrombin found with the two-stage test (% of normal)
0	87
15·6	50
125·0	40
312·5	15

The data given in Table 8 show that the two-stage test yields low prothrombin values in the presence of poly-L-lysine. Thus, the serum prothrombin values given in Tables 3 and 4 are minimal values. The disturbance in thrombin formation caused by the basic poly-amino-acids must be more pronounced than is indicated by the data given.

As it has been proved that poly-lysine in the concentrations used has no effect on fibrinogen, it was of interest to investigate its action on each of the two stages of the two-stage method. It was found that the basic poly-amino-acids in final concentrations up to 300 μg./ml. do not interfere with the second stage, i.e. the titration of thrombin on fibrinogen. The first stage, i.e. the formation of thrombin from prothrombin, on the other hand, is markedly inhibited by poly-lysine and other basic poly-amino-acids in the concentration range used.

Antiheparin activity of poly-L-lysine, poly-DL-lysine and poly-DL-ornithine

In order to study the action of the different poly-amino-acids on heparinized blood, the following experiments were carried out: 1·6 ml. blood were added immediately after venepuncture to a mixture of 0·2 ml. heparin (80 μg./ml.) and 0·2 ml. poly-amino-acid solution (130 μg./ml.) and the clotting time recorded. Blood (1·8 ml.) added to 0·2 ml. heparin (80 μg./ml.) did not clot within 90 min. The clotting time of untreated blood was 7–8 min.

The clotting times observed indicate that the premixing of 16 μg. heparin with approx. 26 μg. of poly-L-lysine hydrochloride, poly-DL-lysine hydrochloride or poly-DL-ornithine hydriodide causes neutralization of the clot-retarding effect of heparin, and restoration of clotting time to normal. The acidic poly-amino-acids, poly-D-glutamic acid and poly-aspartic acid, as well as the neutral poly-α-amino-acid, poly-DL-alanine, had no demonstrable antiheparinic effect in these concentrations. The amino-acid monomers, L-lysine, DL-lysine, DL-ornithine and L-aspartic acid also did not show a significant antiheparinic action.

Neutralizing ratios of heparin to basic poly-α-amino-acids. Anti-poly-lysine activity of heparin

In the previous section it was pointed out that poly-L-lysine, in adequate concentration, may neutralize the clot-retarding activity of heparin. In a series of experiments the effect of mixtures of

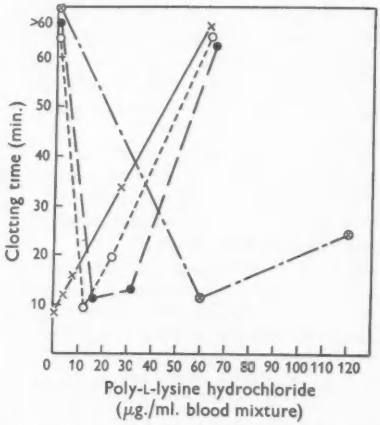

Fig. 1. The effect of mixtures of heparin and poly-L-lysine on the clotting time of human blood. Points representing blood mixtures containing the same concentration of heparin are joined by full or broken lines. Concentration of heparin in μg./ml. blood: –×—×–, 0·0; –○-----○–, 8·0; –●——●–, 20·0; ⊕—·—⊕–, 40·0.

heparin and poly-L-lysine in various concentrations, on blood coagulation was investigated. The experiments were carried out as follows:

1·6 ml. blood was added immediately after venepuncture to a mixture of 0·2 ml. heparin solution (or 0·2 ml. saline) and 0·2 ml. poly-L-lysine solution of various concentrations, and the clotting times recorded. The concentrations of

heparin and poly-L-lysine hydrochloride in the final blood mixtures and the corresponding clotting times are given in Fig. 1.

The clotting times recorded (Fig. 1) suggest that heparin and poly-L-lysine are mutually inactivated when they are present in suitable proportions. It was observed that the mixing of the basic poly-lysine with the acidic heparin leads to the formation of a precipitate, and it may be assumed that both components in the precipitate are rendered inactive. When poly-lysine is added in excess, some of it remains in solution and thus prolongs the clotting time. The antagonistic action of heparin and poly-L-lysine is demonstrable both when they are mixed previously to addition of blood, and when either of the components is added to the system sequentially. The addition of the antagonist in a neutralizing ratio, induced the appearance of a clot within a few minutes, even in blood which had been previously incubated with the coagulation inhibitor (poly-L-lysine, or heparin) for 5–20 min.

The action of acidic poly-α-amino-acids on blood treated with basic poly-α-amino-acids or with protamine

In some of the experiments recorded in Table 9, 1·6 ml. blood were added immediately after venepuncture to a mixture of 0·2 ml. of basic and 0·2 ml. acidic poly-amino-acid solution. In other experiments 1·6 ml. of blood were mixed immediately after venepuncture with 0·2 ml. of the basic poly-amino-acid solution, incubated for 5 min. and subsequently 0·2 ml. of acidic poly-amino-acid was added.

Table 9 shows that the acidic poly-α-amino acids, poly-aspartic acid and poly-D-glutamic acid in the concentration of 120 μg./ml. of blood mixture have no anticoagulant effect; they neutralize the anticoagulant activity of the basic poly-amino-acids. The anticoagulant activity of protamine is also antagonized by poly-aspartic acid.

DISCUSSION

From the data presented it follows that the basic poly-amino-acids, poly-L-lysine, poly-DL-lysine and poly-DL-ornithine, prolong the clotting time of human whole blood, when added immediately after venepuncture. The clot-decelerating activity of the basic poly-amino-acids was found considerably smaller than that of heparin and greater than that of the protamine preparation used. The basic poly-amino-acids also exhibit a distinct antiheparinic effect *in vitro* as demonstrated by the normalization of the clotting time of heparinized blood (Fig. 1).

The neutral water-soluble poly-amino-acid, poly-DL-alanine, and the acidic poly-amino-acids, poly-aspartic acid and poly-D-glutamic acid, showed no effect on blood clotting in the concentration of 120 μg./ml. blood mixture. The presence of basic amino groups in the poly-amino-acids studied seems to be essential for the anticoagulant action. This conclusion is supported by the observation that poly-lysine and poly-ornithine solutions containing equal molar concentrations of free amino groups (equal base mol concentrations) with approximately similar dissociation constants, possess equal anticoagulant activity. The fact that the anticoagulant and antiheparin activities of poly-L-lysine and poly-DL-lysine do not differ significantly indicates that the steric configuration of the amino-acid residues in the active polymer does not play a major part in the determination of the anticoagulant and antiheparin properties.

Since it was found that the corresponding basic amino-acid monomers do not influence blood coagulation and do not act as antiheparins in the investigated range of concentrations, it seems justified to assume that the chain length of the basic poly-amino-acids may be of importance in determining the anticoagulant and antiheparin properties of the basic polymers.

In the study of the mode of blood anticoagulant action of the basic poly-amino-acids, poly-L-lysine was chosen as representative of the group. It was shown that the clot-retarding effect of the poly-L-lysine in the concentrations used is not due to the inactivation of fibrinogen, nor to an increase in antithrombic activity of the blood. It could be demonstrated, that the clot retardation induced was associated with the presence of elevated prothrombin

Table 9. *Action of acidic poly-α-amino-acids on blood treated with basic poly-α-amino-acids or with protamine*

Poly-amino-acid (120 μg./ml.)	Components of test mixture (ml.)*													
Poly-L-lysine hydrochloride	—	0·2	—	0·2	0·2	—	—	—	—	—	—	—	—	—
Poly-DL-ornithine-hydriodide	—	—	—	—	—	0·2	0·2	0·2	—	0·2	0·2	—	—	—
Poly-aspartic acid	—	—	0·2	0·2	**0·2**	—	0·2	**0·2**	—	—	—	—	0·2	**0·2**
Poly-D-glutamic acid	—	—	—	—	—	—	—	—	0·2	0·2	**0·2**	—	—	—
Protamine sulphate	—	—	—	—	—	—	—	—	—	—	—	0·2	0·2	0·2
Saline	0·4	0·2	0·2	—	—	0·2	—	—	0·2	—	—	0·2	—	—
Blood	1·6	1·6	1·6	1·6	1·6	1·6	1·6	1·6	1·6	1·6	1·6	1·6	1·6	1·6
Clotting time (min.)	11	>90	8	11	11	90	14	8	7	20	5·5	22	12	8

* Poly-α-amino-acids other than those shown in black were put into the test tubes previously to the addition of the blood. Components shown in black were added to the test mixtures 5 min. after the addition of the blood.

levels in the serum of the slowly clotting blood. This finding indicates that poly-L-lysine interferes with the formation of thrombin from prothrombin in clotting blood. The experiment performed with thromboplastin (cf. Table 5) indicates that the inhibition of thrombin formation by poly-L-lysine is due to its antithromboplastic action rather than an antiprothrombic action. The possibility has not, however, been excluded that poly-L-lysine also exerts an inhibitory action on the formation of thromboplastin in shed blood, or that it inactivates prothrombin or one of the accelerators of prothrombin conversion (Owren, 1947; Ware, Guest & Seegers, 1947). Inhibition of thrombin formation was also demonstrated with poly-DL-lysine and poly-DL-ornithine; it seems fair to assume that the mechanism of clot-decelerating action of these polymers is similar to that of poly-L-lysine.

The striking similarity of the anticoagulant and antiheparin activities of the basic poly-amino-acids to those of protamines is of interest. Thus, it has been shown by Chargaff (1938*b*) that the action of protamine on blood clotting is at least partially due to the inhibition of thromboplastin. This conclusion was supported by his finding (Chargaff, 1938*a*) that protamine precipitates kephalin in a pH range of 2 to 11, suggesting that the inactivation of thromboplastin by protamine is caused by its reaction with the lipid component of the thromboplastic lipoprotein. As we have found in some preliminary experiments that kephalin is precipitated by the basic poly-amino-acids used in the present work in the pH range of 2–10, the conclusion drawn by Chargaff as to the mode of action of protamine on thromboplastin seems to hold for the action of water-soluble basic poly-amino-acids as well. Inhibition of thrombin formation by protamine has recently also been shown by Portman & Holden (1949). In this connexion, it should be recalled that Mylon, Winternitz & de Sütö-Nagy (1942) and Portmann & Holden (1949) showed that protamine at relatively high concentrations precipitates and inactivates fibrinogen. The latter authors found, however, that at lower concentrations (1 mg. protamine sulphate per ml. blood) which still produce clot retardation, no inactivation of fibrinogen occurs.

Since heparin is precipitated by basic poly-amino-acids as well as by protamine (cf. Jaques, 1943), and since the precipitate formed has no anticoagulant action, it seems likely that inactivation of heparin in heparinized blood results from the formation of an inactive salt between the strongly acidic heparin and the basic poly-amino-acids. Our experiments with mixtures of heparin and poly-L-lysine in various proportions indicate that the anticoagulant activity of the mixtures is determined by the relative concentrations of the components. Excess of either one of the components leads to the prolongation of the clotting time. These results are in accordance with the findings of Jaques (1943) with heparin-protamine mixtures. The similarity between the effect on blood clotting of the synthetic basic poly-amino-acid, poly-L-lysine, with that of a naturally occurring basic protein, protamine (cf. Portmann & Holden, 1949) is of interest and may be summarized as follows: (*a*) Poly-L-lysine and protamine act as anticoagulants on whole blood *in vitro*. (*b*) Both inhibit thrombin formation. (*c*) Both show antithromboplastic activity. (*d*) In the concentrations investigated in the present study leading to prolongation of blood-clotting time, poly-lysine and protamine do not influence the sensitivity of fibrinogen towards thrombin. (*e*) In the effective anticoagulant concentrations studied they do not influence the antithrombic activity of serum. (*f*) Both act as antiheparins. (*g*) The anticoagulant effect of poly-lysine as well as that of protamine is neutralized by heparin and by the acidic poly-amino-acids, poly-aspartic acid and poly-D-glutamic acid.

SUMMARY

1. Synthetic basic poly-α-amino-acids (poly-L-lysine, poly-DL-lysine and poly-DL-ornithine) retard blood clotting markedly in a concentration of about 100 μg./ml. blood. The neutral water-soluble poly-α-amino-acid (poly-DL-alanine) and the acidic poly-α-amino-acids (poly-aspartic acid and poly-D-glutamic acid) show no anticoagulant activity in the concentration range studied.

2. The basic poly-amino-acids inhibit thrombin formation in blood and plasma. Incubation with poly-L-lysine markedly diminishes the ability of thromboplastin to convert prothrombin into thrombin. The sensitivity of fibrinogen to thrombin and the antithrombic activity of serum are not influenced by the basic poly-amino-acids.

3. The clot-retarding action of heparin is neutralized by the basic poly-amino-acids. The neutral and acidic poly-amino-acids do not manifest antiheparin activity.

4. The anticoagulant activity of the basic poly-amino-acids and of protamine may be netralized by heparin as well as by the acidic poly-amino-acids.

5. The similarity of the mode of action of synthetic water-soluble basic poly-amino-acids and of natural protamine on blood coagulation and heparin is pointed out.

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The Isolation of $\alpha\epsilon$-Diaminopimelic Acid from *Corynebacterium diphtheriae* and *Mycobacterium tuberculosis*

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Hydrolysates of *Corynebacterium diphtheriae* have been shown by paper chromatography to contain an unknown neutral α-amino-acid (Work, 1949*a*, 1950*a*). Identical spots on chromatograms were also found by Klungsøyr & Synge (footnote, Work, 1950*b*) using acid hydrolysates of rumen contents of sheep, and by Asselineau, Choucroun & Lederer (1950) using an acid hydrolysate of an antigenic lipopolysaccharide from *Mycobacterium tuberculosis*. Preliminary reports have already been published on the isolation and identification of this amino-acid from *C. diphtheriae* (Work, 1949*b*, 1950*b*); the present paper gives details of its isolation, degradation and identification.

The behaviour of the unknown amino-acid on paper chromatograms has already been fully described ('neutral substance', Work, 1950*a*). Fig. 1 shows its position in relation to the commonly occurring amino-acids on the phenol-collidine two-dimensional chromatogram. The substance gives a spot which is practically identical in position with those given by ethanolamine-*O*-phosphoric acid and cystine. It can be distinguished from cystine by its stability to hydrogen peroxide, and from ethanolaminephosphoric acid by its behaviour during electrodialysis. Since there was no distinctive chemical or biochemical test by which the amino-acid could be recognized, its isolation was only possible through constant use of paper chromatography, each step being followed on two-dimensional chromatograms. The method finally used for the isolation from hydrolysates of *C. diphtheriae* depended on properties already reported (Work, 1950*a*), namely retention with the acidic amino-acids on acid-treated alumina and failure to migrate in an electric field. Acid-treated alumina was shown by Wieland (1942) to act as an anionic exchanger for acidic amino-acids; accordingly, in early attempts to purify the amino-acid, removal of the acidic constituents from the bacterial hydrolysate was investigated using columns of acid-treated alumina. When the amount of acid-treated alumina relative to the total acidic amino-acids of the hydrolysate was roughly of the order used by Wieland, only the acidic amino-acids were retained on the column; but when the proportion of alumina was increased three times, the unknown amino-acid also was retained, while the bases and the other neutral

components of the hydrolysate were washed through. Ammonia eluates from the alumina columns then contained the unknown amino-acid mixed only with acidic substances, and the unknown neutral amino-acid was thus freed from other neutral substances. Separation from the acidic substances in the ammonia eluate was carried out by electrodialysis, the unknown amino-acid remaining immobile while the acidic substances migrated towards the anode. The immobile neutral aqueous fraction was concentrated without further purification and the unknown amino-acid crystallized.

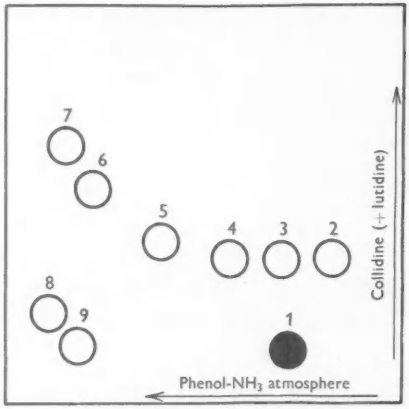


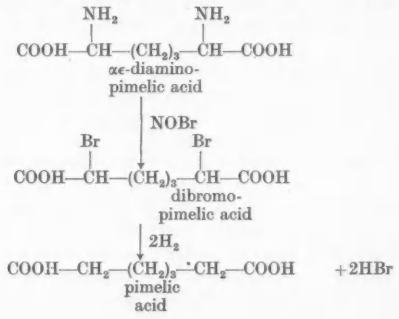
Fig. 1. Position of diaminopimelic acid on phenol-NH_3/collidine-lutidine chromatograms. 1, diaminopimelic acid; 2, aspartic acid; 3, glutamic acid; 4, glycine; 5, alanine; 6, leucine; 7, valine; 8, arginine; 9, lysine.

Identification. The crystalline product, in the form of white needles (Fig. 2), did not melt below 305°. It was soluble in hot water and dilute acids and alkalis, moderately soluble in cold water and insoluble in ethanol and all organic solvents. Elementary analysis gave an empirical formula $C_7H_{14}N_2O_4$, and Van Slyke ninhydrin analysis showed that all the nitrogen was α-amino nitrogen. Although resembling the neutral amino-acids in its behaviour during electrodialysis, the compound exhibited some similarity to cystine and to the acidic amino-acids in its position on paper chromatograms and its retention on acid-treated alumina; it also formed a barium salt insoluble in 50% ethanol. These facts suggested that the substance might be an αα′-diamino-dicarboxylic acid.

All the straight-chain αα′-diamino-dicarboxylic acids $COOH.CH(NH_2).(CH_2)n.CH(NH_2).COOH$ up to diaminosebacic acid ($n=6$) have been synthesized (Neuberg & Neumann, 1905; Neuberg & Federer, 1906; Sørensen & Andersen, 1908; Carter, van Abeele & Rothrock, 1949), but they have not yet been found to occur in nature. With the exception of diaminopimelic acid ($n=3$), these amino-acids are reported to be insoluble in cold water. The properties of diaminopimelic acid (Sørensen & Andersen, 1908) resembled closely those of the unknown substance; this fact, coupled with the elementary analysis, gave reason to assume that the unknown amino-acid might in fact be αε-diaminopimelic acid. Comparison of the naturally occurring amino-acid with synthetic specimens of αγ-diaminoglutaric, αδ-diaminoadipic and αε-diaminopimelic acids strengthened this assumption. Diaminopimelic acid resembled the unknown amino-acid in solubility, crystalline form and chromatographic behaviour, 5 μg. giving a purple spot on a chromatogram indistinguishable from that of the natural material. Diaminoadipic acid was insoluble in water and could not be detected on chromatograms, while diaminoglutaric acid was slightly soluble in water and gave a typical purple colour with ninhydrin, but 50 μg. on a paper chromatogram gave only a weak brown spot well away from that given by the unknown amino-acid (see Work, 1950*a*).

The new amino-acid was converted to the benzoyl derivative, the properties of which were compared with those of authentic αε-dibenzamidopimelic acid. Both compounds were soluble in aqueous ethanol and ethyl acetate. This solubility of both dibenzamidopimelic acid and the free amino-acid were contrasted by Sørensen & Andersen (1908) with the general insolubility of diaminoadipic acid and its dibenzoyl derivative. The benzoylated amino-acid from *C. diphtheriae*, although showing slightly different crystalline form and melting point from those of the synthetic derivative, produced no depression of melting point of the synthetic compound and analysed correctly for dibenzamidopimelic acid. The differences between the crystals of the synthetic and natural derivatives could be accounted for by differences in optical form, since the molecule possesses two asymmetric carbon atoms.

Degradation. Confirmation of the assumption that the natural product was indeed diaminopimelic



acid was obtained by degradation. The amino-acid was brominated with nitrosyl bromide and reduced catalytically; the product was identified as pimelic acid by its melting point and by the fact that it did not depress the melting point of an authentic specimen of pimelic acid. As the Van Slyke analysis had already demonstrated that all the nitrogen of the molecule was in the form of α-amino nitrogen, the only possible structure for the compound is αε-diaminopimelic acid.

Optical form. Diaminopimelic acid isolated from *C. diphtheriae* was found to be optically inactive. The compound is probably the internally compensated meso-isomer in which one α-carbon atom has the configuration of D-alanine and the other α-carbon atom that of L-alanine, since the method of preparation was such as to render unlikely the alternative explanation of optical inactivity, i.e. racemization. Four separate batches of material were examined for optical activity, in one of which ammonia was removed by ion exchange instead of vacuum distillation; the possibility seems remote that racemization should have occurred in all four preparations. However, proof of optical form must await resolution of synthetic material.

Dr K. Burton has examined the behaviour of natural diaminopimelic acid towards the L-amino-acid oxidase of *Neurospora crassa* (see Bender & Krebs, 1950) and reports as follows: 'αε-Diaminopimelic acid is readily oxidized by the L-amino-acid oxidase of *N. crassa* with the consumption of one atom of oxygen and the liberation of one molecule of ammonia/molecule of amino-acid added. The rate of oxidation is about the same as that of L-phenylalanine. The quantities of oxygen absorbed and of ammonia produced correspond to the oxidation of half the amino groups to keto groups. This result could be explained by the assumption that the diamino-acid was a racemate. However, the ninhydrin colour reaction became negative, indicating that both α-amino groups had disappeared. No 2:4-dinitrophenylhydrazone was detectable on addition of the hydrazine, indicating that no ketone was formed. A possible explanation of these results is the assumption that α-keto-ε-aminopimelic acid is primarily formed and undergoes secondary ring enclosure to give a tetrahydropyridine derivative (cf. Gabriel, 1908; Lipp, 1896).' Dr E. Bonetti (private communication) found that natural diaminopimelic acid was not oxidized by either ophio L-amino-acid oxidase or kidney D-amino-acid oxidase.

Natural distribution. Since Klungsøyr & Synge and Asselineau *et al.* (1950) also found spots matching diaminopimelic acid in chromatograms from other substances of bacterial origin, it was of interest to examine the distribution of the new amino-acid among micro-organisms. Work on this aspect is in progress and will be fully reported elsewhere, but it can be said here that, to date, the amino-acid has been identified in chromatograms from some twelve bacterial species including *M. tuberculosis* (human, bovine, B.C.G. and avian strains), but has not been found in all bacteria examined, neither has it been found in any product of non-bacterial origin. Crystalline diaminopimelic acid was also isolated from *M. tuberculosis* (human strain), thus confirming the chromatographic identification of the amino-acid in this organism.

The concentration of diaminopimelic acid in different bacteria is variable, but it appears that the amino-acid is by no means a minor constituent. In ethanol-extracted *C. diphtheriae*, for example, diaminopimelic acid accounts for about 2% of the insoluble cell nitrogen, and thus equals the concentration of many other amino-acids in this organism; a small amount was also found with the extractable amino-acids in the ethanolic extract (Work, 1949*a*, 1950*a*). The amino-acid was found neither in tuberculin (see also Asselineau *et al.* 1950), nor in diphtheria toxin, both exogenous proteins produced by organisms known themselves to contain diaminopimelic acid.

EXPERIMENTAL

Isolation of diaminopimelic acid from Corynebacterium diphtheriae

Material. Dried *C. diphtheriae* (P.W. 8 strain) (extracted with 65% (v/v) ethanol, Work, 1949*a*, or unextracted) was hydrolysed for 24 hr. under reflux with 6N-HCl (800 ml./100 g. dry wt.). The hydrolysate was evaporated to dryness *in vacuo*, redissolved in water and dried *in vacuo*; this was repeated three times, but before the last evaporation, humin was removed by filtration and washing with hot water. The residue was dissolved in water (3 ml./1 g. of dried cells).

Alumina columns. Alumina (Savory & Moore, for adsorption purposes) was converted to the acid form by stirring for 15 min. with N-HCl (3 l./kg. Al_2O_3) and washing by percolation with water overnight until the washings had pH 5–6 (Wieland, 1942). Four columns, 4·5 cm. in width and 60–80 cm. long, were packed with acid-treated Al_2O_3 (1 or 1·5 kg. according to length) in water, and were allowed to settle over 24 hr. During all subsequent operations, percolation through the columns was speeded up by maintaining a nitrogen pressure equivalent to 15 cm. Hg above the Al_2O_3. After removing the supernatant water from the columns, neutralized bacterial hydrolysate (50 ml. on the 1·5 kg. columns and 33 ml. on the 1 kg. columns, Work, 1950*a*) was run into the columns, which were washed with water (7–8 l.) until the percolate was substantially free from amino-acids (no ninhydrin colour produced by 3 drops dried successively on filter paper). The percolate was discarded and the columns were eluted with N-NH_3 solution.

Each column before elution had a brown band 2–3 cm. in width at the top, a narrow pink band about 10 cm. down, with sometimes a narrow yellow band between the two; the rest of the column was white. The pink band moved just behind the NH_3 front, and collection of eluate was started

when this band reached the bottom of the column and the percolate became pink and alkaline. The percolate just before the pink band contained the bulk of the Cl^- which had remained on the column, but Cl^- could not be completely eliminated from the eluate owing to the fact that the amino-acids commenced to come off with the pink band, when some Cl^- was still present. It was not possible to separate diaminopimelic acid from the acidic amino-acids as all came off the column together throughout the elution. Elution was continued until 3 drops of percolate on filter paper no longer gave a positive ninhydrin reaction (7–8 l.).

The eluate was concentrated and freed from NH_3 by vacuum distillation, with bath temperature below 60°. NH_3 was removed by ion exchange prior to concentration from one batch of eluate using Amberlite IRC/50 (Resinous Products and Chemical Co., U.S.A.), buffered at pH 4·7 with M-acetate buffer. This batch was worked up separately.

The columns were regenerated *in situ* by passing N-HCl through until the percolate was acidic and slightly cloudy, after which they were washed with water until the percoiate had pH of between 5 and 6 (about 10 l.); re-cycling could be continued indefinitely.

Electrodialysis. A four-compartment cell of 14 cm. diam. Perspex tubing with two inner compartments of width 2·5 cm. and end compartments of width 3·5 cm. was used. Thin platinum wire electrodes (0·01 in. (0·25 mm.) diam. and 3 ft. (91·5 cm.) length) were attached to Perspex end plates, on the opposite side of which cooling water was passed. The centre compartments were not cooled, as the main heating occurred at the anode and cathode. Formalized parchment (Campbell, Work & Mellanby, 1951) was used for the semi-permeable membranes separating the cells, as it allowed passage of the acidic amino-acids to the anode much faster than did cellophan.

Concentrated filtered eluate (equiv. to 70 g. dried cells) was placed in the compartment (subsequently called 'centre') next to the cathode, other compartments were filled with water and a potential difference of 220 V. was applied to the electrodes. The current rose rapidly and was maintained at 1·0 amp. by adjusting the voltage. As chloride migrated to the anode, the contents of which were replaced by water frequently, the current stayed constant and finally fell slowly to about 0·02 amp. The pH of the centre compartment was recorded, but not regulated, since diaminopimelic acid remained immobile under all pH conditions provided there were no basic amino-acids moving to the cathode (see Work, 1950*a*). At the beginning, the pH was about 2·5–3; once all the Cl^- had reached the anode, leaving the compartment next to the anode less acidic, the acidic amino-acids migrated to this compartment, accompanied by most of the colour originally present and the 'centre' compartment reached neutrality. The electrodialysis was continued for several hours after the current had reached the minimum value, the total time being 24–30 hr. Occasionally the pH of the centre compartment rose as high as 10, while the catholyte was less alkaline than usual and the current dropped unusually quickly; a dark coating was found on the cathode electrode and was removed by washing with dilute acid. Because of this periodic coating of the cathode wire, it was rinsed with dilute acid between every batch.

The pale-yellow liquid from the 'centre' compartment was chromatographed on paper to ensure that diaminopimelic acid was the only amino-acid present. If any aspartic or glutamic acid were detected, the solution was electrodialysed for a further period of 5 or 6 hr.

Crystallization. The liquid from the 'centre' compartment was decolorized (charcoal) and concentrated until the hot solution was milky in appearance; on cooling, a large mass of crystals of diaminopimelic acid appeared. To recrystallize the material, it was necessary to dissolve in about four times the volume of water from which it originally crystallized and then concentrate. Crystallization could also be effected by addition of ethanol to a concentrated aqueous solution until a faint cloudiness appeared. Material for analysis was crystallized twice from water and once from aqueous ethanol.

Yield. The average yield from many batches of hydrolysate was 1 g. of crude diaminopimelic acid from 150 g. of dried, extracted *C. diphtheriae* cell residues. This represents 40% of the expected yield, since the hydrolysed ethanol-extracted cells contained approx. 2% of their total N as diaminopimelic acid (estimated by paper chromatography, Work, 1950*a*; Lindan & Work, 1951).

Properties of the natural amino-acid

Diaminopimelic acid crystallized from water as fine white needles (Fig. 2) and its hydrochloride as elongated hexagonal plates; the free amino-acid did not melt below 305°, the hydrochloride decomposed about 240°. The free amino-acid was found to be freely soluble in dilute acids and alkalis but to dissolve only with difficulty in water even on heating; at 21° a saturated solution was found to contain 0·92% (w/v) of the amino-acid. Diaminopimelic acid formed a Ba salt insoluble in 50% (v/v) ethanol: precipitation as the Ba salt was a useful method for recovery from crude mother liquors.

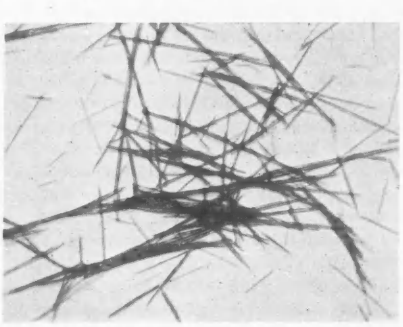


Fig. 2. Crystals of diaminopimelic acid from *C. diphtheriae* (×380).

No optical rotation was observed for diaminopimelic acid in 2·75% (w/v) solutions in 6N-HCl, 12N-HCl or 2N-NaOH (2 dm. tube). Four separate batches prepared at different times were examined, from one of which NH_3 had been removed by ion exchange prior to vacuum distillation.

Identification of the natural amino-acid as αε-diaminopimelic acid

The following analytical figures were obtained: C, 44·3; H, 7·45; N, 14·8. $C_7H_{14}N_2O_4$ requires C, 44·2; H, 7·42; N, 14·8%. α-Amino N, 14·4% (ninhydrin-CO_2 method, pH 2·5; Van Slyke, Dillon, MacFadyen & Hamilton, 1941), i.e. all N present as α-amino N. The free amino-acid was not sufficiently soluble in camphor or water for molecular weight determination.

Dibenzamidopimelic acid. Natural diaminopimelic acid (40 mg.) was dissolved in 1 ml. of 0·5N-NaOH, and three separate additions were made of benzoyl chloride (total 0·28 g.) followed immediately by 2N-NaOH (total 1·8 ml.), with cooling and shaking for about 5 min. after each addition (Sørensen & Andersen, 1908). After standing 10 min. the mixture was filtered, acidified to pH 2–3 with 3N-HCl and kept at 0° for 30 min.; the precipitate was filtered off, washed with water, dried *in vacuo* over H_2SO_4 and thoroughly washed with light petroleum to remove benzoic acid. The crude product weighed 77 mg. (yield 87% of theoretical). Two crystallizations from 25% (v/v) ethanol-water produced a mixture of plates and rosettes; m.p. 181–199° (microstage) unchanged on further recrystallization from the same solvent. Subsequent crystallization from ethyl acetate-ligroin produced fine needles, m.p. 193° (capillary tube). (Found: C, 63·7; H, 5·9; N, 6·9. $C_{21}H_{22}N_2O_6$ requires C, 63·3; H, 5·6; N, 7·0%.) Synthetic dibenzamidopimelic acid from Prof. K. Linderstrøm-Lang also melted at 181–199° on the microstage; recrystallization from 25% ethanol and from ethyl acetate-ligroin produced inhomogeneous crystals, m.p. 195–197° (capillary tube), mixed m.p. with natural dibenzoyl derivative 195°.

Degradation to pimelic acid. Pure natural diaminopimelic acid (107 mg.) was dissolved in 3 ml. 2N-H_2SO_4 containing 392 mg. KBr, and $NaNO_2$ (152 mg.) was added with cooling in small portions over 1·75 hr. (Neuberger & Sanger, 1944). Violent frothing with brown fumes occurred after each addition; after 30 min. the solution turned pale green, and a white solid crystallized out. When additions were completed the mixture stood at 0° for a further 30 min., until most of the bubbling had stopped, and after aeration (N_2) for 30 min., was extracted with ether in a continuous extractor. The dried ethereal extract yielded 150 mg. of oily material which could not be crystallized, but was fractionated by dry ether into two portions. The dry ether-soluble fraction (104 mg.) dissolved in 50% (v/v) ethanol, was hydrogenated in alkaline solution at room temperature and atmospheric pressure with Pt black as catalyst. H_2 uptake was 100% of theoretical for 104 mg. dibromopimelic acid. After acidification, the solution was extracted with ether in a continuous extractor; the dried ethereal extract yielded a waxy solid (50 mg.) which was crystallized three times from dry ether, m.p. 102° (microstage) (pimelic acid, m.p. 104°; mixed m.p. with degradation product 103°), overall yield 55% theoretical.

Identification of diaminopimelic acid in Mycobacterium tuberculosis

Dried steam-killed *M. tuberculosis* of human, B.C.G., bovine and avian strains were each hydrolysed as described for *C. diphtheriae*. Paper chromatograms of the hydrolysates (equiv. to 3 mg. dried cells) showed the diaminopimelic acid spot in all cases. Each hydrolysate was electrodialysed in a small three-compartment cell (capacity of centre 10 ml.) and samples (equiv. to 3 mg. dry cells) of the centre compartment were chromatogramed after treatment with H_2O_2 and ammonium molybdate (Dent, 1948). Diaminopimelic acid spots were found on all chromatograms.

Diaminopimelic acid (Found: C, 43·9; H, 7·3; N, 14·5%) was isolated from hydrolysates of *M. tuberculosis* (human strain), the method of preparation being identical to that used in the case of *C. diphtheriae*.

Examination of tuberculin and diphtheria toxin for diaminopimelic acid

Chromatograms of hydrolysates of tuberculin and diphtheria toxin (untreated and toxoided) showed no diaminopimelic acid spot, even when more than 1 mg. of starting material was used. With this large amount of material, the glutamic acid spots were so large as to obscure any weak diaminopimelic acid spots, so the hydrolysates were electrodialysed and the neutral fraction put on chromatograms in amounts up to 6 mg. of starting material. Diaminopimelic acid was not found.

DISCUSSION

Asselineau *et al.* (1950) reported that diaminopimelic acid was absent from defatted cells of *M. tuberculosis* (human strain) and from tuberculin and present only in the ether-soluble antigenic lipopolysaccharide fraction, which contained in addition only two other amino-acids, alanine and glutamic acid. I have confirmed the absence of diaminopimelic acid from tuberculin, but, having isolated the amino-acid from the human strain of *M. tuberculosis*, suggest that the antigenic lipopolysaccharide (comprising only 7·6% of the total cells) could not be contributing the whole content. A later report by Asselineau & Lederer (1950) showed that diaminopimelic acid is only present in the lipopolysaccharide from virulent strains, being absent from the fraction from B.C.G. strain; since I found the amino-acid to be present in whole cells of B.C.G. strain, it must occur in some cell constituents other than the extractable lipopolysaccharide.

The failure of Asselineau *et al.* to find diaminopimelic acid in chromatograms of *M. tuberculosis* cells may be attributed to the fact that the detection of the amino-acid in chromatograms from crude cell hydrolysates is sometimes difficult because it is directly below the glutamic acid spot, which sometimes may be so large as to obliterate or fuse into the weaker diaminopimelic acid spot. When examining a material for diaminopimelic acid, it is advisable to electrodialyse it, then to examine chromatograms of the neutral fraction. Electrodialysis removes the acidic amino-acids, thus preventing obliteration by glutamic acid; it will also prevent diaminopimelic acid from being confused with ethanolamine-*O*-phosphoric acid, which runs identically on phenol-collidine chromatograms. Confusion with sulphur-containing amino-acids can be prevented by investigating stability of the neutral spot to hydrogen peroxide (Work, 1950*a*).

A figure for the diaminopimelic acid content of ethanol-extracted *C. diphtheriae* has been arrived at, using paper chromatography as a means of estimation. This method is admittedly rough and unless certain precautions are followed (Lindan & Work, 1951) can give very unreliable figures. This accounts for the high value of 4–8% of the total nitrogen

originally reported (Work, 1949*b*); the figure is now calculated to be about 2%.

Diaminopimelic acid is the first diamino-dicarboxylic acid, other than cystine, to be identified in nature. In this connexion it should be pointed out that the presence of its lower homologues diaminoglutaric and diaminoadipic acids would be much harder to demonstrate in view of their insolubility and the probable difficulty of identifying them by paper chromatography. Sørensen & Andersen (1908) report that diaminoadipic acid is soluble only in 5N-HCl, so, although it would be in solution in 6N-HCl hydrolysates, it would precipitate as soon as the HCl was removed. Diaminoglutaric acid, although soluble in mineral acids, does not give a well defined spot on chromatograms. The literature contains little information on the chemistry of $\alpha\alpha'$-diaminodicarboxylic acids, and no explanation has been advanced for the poor solubility in water of all the members of the series except for diaminopimelic acid. Spontaneous ring closure to double lactams or polymerization to polyamides could account for the absence of typical spots on chromatograms of diaminoglutaric and diaminoadipic acids. This lactamization could occur either during preparation or on the paper and would lead to a negative ninhydrin reaction, since such 5- or 6-membered rings would not contain free amino groups. Lactamization could not, however, explain the insolubility of the substances. It is interesting to note that $\beta\gamma$-diaminoadipic acid and its lactam are water-soluble (Traube, 1902). If insolubility were due to polymerization, it is difficult to understand why diaminopimelic acid is not also insoluble; by analogy with the ω-monoamino-monocarboxylic acids, one would expect diaminopimelic acid to polymerize more easily than its two lower homologues, since in the monoamino-acid series, γ-aminobutyric acid and δ-aminovaleric acids lactamize fairly completely on heating, while ϵ-aminocaproic acid (the monoamino-acid corresponding to diaminopimelic acid) tends to polymerize rather than to lactamize to a 7-membered ring (Carothers, 1931). The higher homologues among the diaminodicarboxylic acids are not as insoluble as the lower homologues, being soluble in dilute acids and alkalis.

The low yields of diaminopimelic acid from *C. diphtheriae* might be due to a certain amount of polymerization during preparation. The appearance of a dark humin-like precipitate was often noted, and even during the acid hydrolysis of synthetic dibenzamidopimelic acid to the free amino-acid, a similar precipitate appeared.

If diaminopimelic acid, as isolated from *C. diphtheriae*, proves in fact to be the internally compensated form suggested by its optical inactivity, it contains one carbon atom of the configuration hitherto regarded as unnatural. Since definite confirmation of the optical form has not yet been obtained, speculation of function in relation to optical configuration is premature. The oxidation by *Neurospora* L-amino-acid oxidase with complete disappearance of ninhydrin reaction, although not proving the meso structure, does indicate that the compound is not the racemate, since in this case only part of the starting material would be oxidized, and the remaining unchanged compound in which both α-carbon atoms have the D-configuration would give the ninhydrin reaction. The lack of oxidation by ophio-L-amino-acid oxidase and kidney D-amino-acid oxidase as opposed to the rapid oxidation by *Neurospora* L-amino-acid oxidase is not surprising, since diaminopimelic acid appears to be confined to micro-organisms, and so might be expected to be unattacked by enzymes of animal origin.

The function and metabolic significance of diaminopimelic acid are at present unknown. Since it did not replace pimelic acid for an exacting strain of *C. diphtheriae* (Hale, private communication), it evidently plays no part in biotin synthesis. It occurs in more than trace amounts, so it may not be a metabolite associated only with synthesis of a trace coenzyme; the fact that its concentration in the insoluble portion of bacteria is of the same order as that of many commonly occurring amino-acids suggests that it might be a constituent of bacterial protein. As diaminopimelic acid has been found so far only in products of bacterial origin, it is of particular interest in being one of those few amino-acids confined to one class of living matter. As such, it might provide a basis for a new line of chemotherapeutic attack on bacteria; if its metabolic path is found to be of importance for growth or pathogenicity, drugs might be designed capable of attacking bacteria through this path and such drug would be non-toxic to a host lacking the amino-acid in its make-up.

SUMMARY

1. An unknown amino-acid appearing on paper chromatograms of hydrolysates of *Corynebacterium diphtheriae* was isolated by means of adsorption from crude hydrolysates on excess acid-treated alumina, followed by electrodialysis of the ammonia eluates.

2. The amino-acid was shown by analysis, degradation and comparison with the synthetic product to be $\alpha\epsilon$-diaminopimelic acid.

3. The natural amino-acid had no optical activity and may be the internally compensated meso-isomer.

4. Diaminopimelic acid was also found in hydrolysates of four strains of *Mycobacterium tuberculosis*, but not in tuberculin. It was absent from diphtheria toxin.

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Paterson, diphtheria toxin and toxoid from Dr G. R. Tristram and Dr L. B. Holt, respectively, diaminoadipic acid and dibenzamidopimelic acid from Prof. K. Linderstrøm-Lang and diaminoglutaric acid from Mr H. Heath. I am indebted to Dr J. H. Hale for growth tests on *C. diphtheriae*, to Dr E. Bonetti and Dr K. Burton for tests with amino-acid oxidases and to Mr J. Smiles for photomicrographs. Drs Weiler and Strauss carried out micro-analyses, Mr A. W. Hemmings constructed electrodialysis apparatus and Mr F. Grover gave valuable technical assistance.

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End-Group Assay in some Proteins of the Keratin-Myosin Group

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The fluorodinitrobenzene (FDNB) method for the detection and estimation of amino groups in proteins (Sanger, 1945) has been applied for the most part to proteins of the soluble corpuscular type. The present study extends the technique to the soluble asymmetric proteins tropomyosin, myosin and fibrinogen, which possess a common intramolecular structure similar to that of α-keratin. For reasons which will be discussed later, the results for fibrinogen are still tentative, and most interest centres at present upon tropomyosin and myosin, which are being studied collaterally from a physico-chemical standpoint (Tsao, Bailey & Adair, 1951). The interpretation of the chemical data is indeed very dependent upon other information, the purity of the protein, its particle weight, and especially upon independent evidence for the existence of sub-units. The difficulty of interpretation arises from the fact that tropomyosin and myosin appear to be built of cyclic polypeptide chains, and the small amounts of terminal amino-acids discovered when dinitrophenyl-protein (DNP-protein) is hydrolysed may be due to very tenacious impurities. Sanger's method in fact, if properly applied, is likely to become the most searching of all tests in the determination of protein purity, and its application has so far been too limited to decide the degree of chemical heterogeneity or contamination in other 'pure' proteins.

MATERIALS AND METHODS

Protein preparations

Ox fibrinogen. The fibrinogen was precipitated from fresh citrated ox plasma by the addition of an equal volume of 2 M-potassium phosphate buffer, pH 6·6 (Jaques, 1943). It was dissolved and reprecipitated four times, and stored frozen as a solution in 0·25 M-phosphate buffer. Fibrin was prepared by diluting with 2 vol. of water and incubating with a trace of purified thrombin for 2 hr. at 25°. The clot was squeezed, dried and ground in ethanol. The amount of non-clottable material was only 5%.

Rabbit myosin. Preparations *SG*1 and *SG*2 (Tables 1 and 3) were the myosin A of Szent-Györgyi (1947) four times precipitated. Preparations *D*1–*D*3 (Table 4) represent a fraction of myosin A precipitating between 40 and 50% saturation with $(NH_4)_2SO_4$ (Dubuisson, 1946, modified by Szent-Györgyi, 1947). Osmotically, this fraction behaves differently from myosin A itself and is thought to be free of actin (Tsao, 1950).

Rabbit tropomyosin. The original method of Bailey (1948) has been only slightly modified. The chief impurity appears to be a colloidal denatured material. Its removal is facilitated by precipitating the tropomyosin from neutral solution with ethanol and drying in ethanol and ether. The protein is then dissolved in water, dialysed against 0·1 M-KCl, and the aggregated protein spun down. The ethanol treatment and dialysis are repeated once more, when a smaller amount of impurity is separated, leaving a clear supernatant liquid.

Treatment with fluorodinitrobenzene

Fibrinogen. In some cases the soluble native protein was treated with FDNB under the conditions which Sanger (1945) recommends; in other cases, the protein was precipitated with ethanol, dried and ground before treatment with the reagent. Such preparations are comparable in physical state with those of fibrin.

Myosin and tropomyosin. To conserve reagent, dilute solutions of these proteins were first treated with M-sodium acetate buffer (pH 5). The precipitated protein was spun down, and brought into solution by addition of solid $NaHCO_3$.

Separation of DNP-amino-acids

General. The DNP-proteins were hydrolysed for various times and under varying conditions to meet the requirements for the detection of the more acid-labile DNP-amino-acids as well as those which are relatively stable, as described by Porter & Sanger (1948). The ether-soluble DNP-acids were first investigated on silica gel-$CHCl_3$ columns, which separate the fast-moving artifacts such as 2:4-dinitroaniline. Characterization of discrete bands on these chromatograms was carried out on other silica columns using a variety of solvents as ambient phase (Sanger, 1945).

ϵ-*N*-DNP-lysine, which remains in the aqueous phase after shaking out the ether-soluble DNP-acids, was quantitatively estimated by evaporating down the solution, extracting with methyl ethyl ketone (66% by vol.)-ether, and passing through a column of an acid gel (1 part silica to 1 part N-HCl), on which the compound is more stable than on neutral gel (Porter, 1950). It was estimated spectrophotometrically at 390 mμ. after dissolving in N-HCl. Ether-soluble DNP-amino-acids, on the other hand, were first dissolved in 1% (w/v) aqueous $NaHCO_3$ and read at 350 mμ. (see Sanger, 1949).

Identification of α-N-*DNP-arginine in the water-soluble fraction.* DNP-arginine and bis-DNP-histidine, if present, would remain with ϵ-*N*-DNP-lysine in the aqueous phase. With methyl ethyl ketone-ether, the band rates of the lysine and arginine derivatives are rather close, so that the latter is likely to be masked by the former which is usually present in large amounts. It was thought that a better separation of these two components might be achieved by suppressing the dissociation of the lysine α-amino group. This was achieved by the incorporation of formaldehyde into the silica gel: 100 g. silica (9% moisture) were mixed with 40 ml. of 1:9 (by vol.) B.P. formaldehyde-water, first neutralized to pH 7 with 0·5 M-Na_2HPO_4. ϵ-*N*-DNP-lysine, dissolved in methyl ethyl ketone-ether, at first penetrates the gel slowly, but as it reacts with formaldehyde, proceeds to run fast: *R* of ϵ-*N*-DNP-lysine and bis-DNP-histidine > 1; DNP-arginine, 0·3. An amount of α-DNP-arginine just visible by itself on a column can be recovered after mixing with 50 mol. of ϵ-*N*-DNP-lysine.

Amide determinations

Air-equilibrated DNP-proteins generally contain about 70% of protein, when allowance is made both for water and DNP content. When the absolute protein weight had to be known accurately, as in the estimation of ϵ-*N*-DNP-lysine, the amide N of the sample was determined and compared with that of the pure protein. The general procedure was similar to that of Bailey (1937).

RESULTS

The reactivity of lysine ϵ-N-*amino groups*

Porter (1948) has reported that a fraction of the lysine side chains in some proteins is unreactive unless the protein is first denatured. On the other hand, the reactivity of terminal amino groups is the same in native proteins as in the denatured. The amounts of ϵ-*N*-DNP-lysine found in fibrinogen, fibrin, tropomyosin and myosin are listed in Table 1. In the case of fibrinogen and fibrin, whatever the pretreatment of the protein before reaction with FDNB, the amount of lysine determined is less than that found on complete hydrolysis with acid. The interesting feature is that the reactive side chains are consistently fewer in fibrin than in fibrinogen, but when the fibrin clot is redissolved in urea and treated with FDNB, the reactivity matches that of fibrinogen. Mihalyi & Lorand (1948) have suggested that amino groups are important in the clotting process, and the present results support the view that the unreactive lysine side chains may play some part in the aggregation of fibrin particles.

Table 1. ϵ-N-*Dinitrophenyllysine from various dinitrophenyl-proteins*

Protein	Sample	Conditions before treatment with fluorodinitrobenzene	Lysine N as % of protein N: As ϵ-*N*-DNP-lysine	Lysine N as % of protein N: Other methods of analysis*
Ox fibrinogen	*KB*	Precipitated with ethanol	8·4	
	KB	8 hr. at 0° in N-HCl	8·4	
Ox fibrin	*KB*	Dried in ethanol	7·3	9·6–10·3
Ox fibrinogen	*KB-IMG*	Precipitated with ethanol	8·6, 8·9†	
Ox fibrin	*KB-IMG*	Dried in ethanol	7·7, 7·8†	
	KB-IMG	Dissolved in conc. urea	8·7	
Myosin	*SG*1	Precipitated at pH 5	13·2	13·7
	*SG*1	8 hr. at 0° in N-HCl	14·0	
Tropomyosin		Precipitated at pH 5	17·1, 16·4†	18·0
		Dissolved in guanidine hydrochloride	19·5, 18·6†	

* See Bailey (1944, 1948).

† Each value is derived from the analysis of separately prepared hydrolysates.

The ε-*N*-DNP-lysine found in DNP-myosin, whether this latter is prepared from native or denatured material, agrees with the value obtained by conventional analysis. In tropomyosin, however, full reactivity is attained only in solvents such as guanidine hydrochloride, and some 15 % of the total lysine appears to be unreactive in the native form.

Because of destructive loss on hydrolysis of DNP-protein, and for other reasons, the absolute accuracy of the DNP-method is not very high. Duplicate analyses on the same hydrolysate, using the same gel, will agree within 5 %. In assessing differences of reactivity such as those reported above, it is important to hydrolyse the proteins side by side and to perform all other operations in parallel. Differences of 15 % are then significant.

Water-soluble DNP-amino-acids other than ε-N-DNP-lysine

In general, the water-soluble DNP-acids were dissolved in methyl ethyl ketone-ether and run on two types of column: (*a*) the acid silica column on which bis-DNP-histidine travels fast, and (*b*) on the silica-formaldehyde column upon which only DNP-arginine runs slowly. Neither histidine nor arginine has been detected as terminal group in myosin and tropomyosin; the investigation of fibrinogen has been deferred.

Ether-soluble DNP-amino-acids

Fibrinogen. Making an approximate correction for hydrolytic destruction, fibrinogen contains one terminal residue of tyrosine/200,000 g. of protein and one of glutamic acid in about 300,000 g. of protein. It is not known whether the fibrinogen molecule, which has a molecular weight of about 500,000 (Adair, see Bailey, 1944; Edsall, Foster & Scheinberg, 1947), contains sub-units to which these terminal residues could be assigned. Moreover, Morrison, Edsall & Miller (1948) have shown that fibrinogen may be contaminated with the so-called 'cold-insoluble globulin', whose relation to fibrinogen remains somewhat obscure. The studies of Abrams, Cohen & Meyer (1949) strongly suggest that the cryoglobulin component arises from pathological plasma which may fortuitously have been admitted into the pooled samples of normal plasma. In the light of these considerations, it would be unwise to interpret the results at the present time.

Tropomyosin. The physico-chemical evidence presented in the accompanying paper (Tsao *et al.* 1951) suggests that the limiting value of the particle weight of tropomyosin, whether native or in depolymerizing solvents, is 53,000; it suggests moreover, almost unambiguously, that the molecule consists of two chains lying side by side in the α-configuration. The results of end-group assay, shown in Table 2, support the idea that the double chain is in fact part of one single continuous polypeptide containing no end groups, i.e. the unit is a cyclic polypeptide.

In impure specimens of tropomyosin, containing visible colloidal material (see p. 23) at least four types of terminal residue occur: one gives an uncharacterized band which is stationary on chloroform gel; there is another band consisting almost entirely of bis-DNP-lysine; finally, there is a faster band consisting mostly of DNP-valine and a little DNP-alanine. In tropomyosin preparations, which have been considered pure by physical tests, the first two bands almost disappear and the valine band remains the strongest. The total colour, however, represents only one terminal residue in 40–50 × 10^4 g. of protein, or one residue in 9 mol. of tropomyosin. Appropriate tests for acid-labile DNP-acids such as glycine and proline have not revealed bands which are different, qualitatively or quantitatively, from those in Table 2. It seems probable, therefore, that the ether-soluble DNP-amino-acids arise from the impurities common to the obviously impure and the 'pure' preparations. If these impurities contained terminal residues to the extent found in insulin, they would amount to a total impurity of less than 1 %.

Table 2 also shows that no peptide bonds are split when tropomyosin is kept at pH 12 and 0° for 14 days, and the same is true with acid conditions at pH 2.

Table 2. *Ether-soluble dinitrophenyl-amino-acids from tropomyosin*

Band character on $CHCl_3$ column	Estimated breakdown (18 hr. hydrolysis)* (%)	Estimated weight of protein containing 1 mol. α-DNP-amino-acid after correction for hydrolytic breakdown			
		Impure tropomyosin 1	Impure tropomyosin 2	Pure tropomyosin	Tropomyosin after 14 days at 0°, pH 12
Stationary	40	320,000	350,000	1,710,000	1,630,000
R 0·2 (bis-DNP-lysine)	10	1,070,000	1,160,000	2,110,000	1,730,000
R 0·5 (DNP-valine, DNP-alanine)	30	290,000	290,000	700,000	1,070,000
Total		112,000†	139,000	403,000	470,000

* Cf. Porter & Sanger (1948).

† Identical recovery on the $CHCl_3$-buffer *B* column of Blackburn (1949).

Table 3. *Ether-soluble dinitrophenyl-amino-acids from myosin*

(Figures in parenthesis are the estimated percentage breakdown appropriate to the conditions of hydrolysis (see Porter & Sanger, 1948).)

Band character on $CHCl_3$ column	Estimated weight of protein containing 1 mol. DNP-amino-acid after correction for hydrolytic breakdown: Myosin *SG*1 (16 hr. hydrolysis)	Myosin *SG*1 (4 hr. hydrolysis)	Myosin *SG*1 (hydrolysis in sealed tube)
Stationary and slow (*R* 0·08)	1,120,000 (40)	3,570,000 (nil)	960,000 (30)
R 0·2 (mainly bis-DNP-lysine)	4,900,000 (10)	6,270,000 (nil)	2,400,000 (30)
R 0·4 (DNP-alanine, DNP-valine)	4,100,000 (30)	2,610,000 (nil)	2,400,000 (20)
Total	740,000	1,200,000	535,000

Myosin. Like tropomyosin, DNP-myosin gives only traces of ether-soluble DNP-acids (Table 3). Again there is an unidentified band stationary on chloroform columns, a band of bis-DNP-lysine and one consisting mainly of DNP-alanine with a little DNP-valine.

Portzehl & Weber (see Weber, 1950) have recently reported that the particle weight of myosin from sedimentation-diffusion and also from osmotic pressure measurements is $8{\cdot}3 \times 10^5$. The number of end groups discovered, supposing them to be homogeneous, amount to no more than one residue/mol. For our present purpose, however, the particle weight is less significant than that of the sub-units of which myosin is composed. Weber & Stöver (1933)

Table 4. *Ether-soluble dinitrophenyl-amino-acids on myosins subjected to alkaline conditions before treatment with fluorodinitrobenzene*

Sample		Estimated weight of protein containing 1 mol. DNP-amino-acid after correction for hydrolytic breakdown
Myosin *D*1*	Treated at pH 13 for 26 days at 0°	156,000
Myosin *D*2	Native protein	420,000
	Treated pH 10·5–11 for 10 days at 0°	520,000
Myosin *D*3	Native protein	360,000
	Treated at pH 13 for 30 days at 0°	37,000

* No control on the native protein.

were the first to investigate the depolymerization of myosin (actually actomyosin) in urea. More recently, Snellman & Erdös (1948) found that when myosin was first dissolved in urea, the solution remained monodisperse although the molecules became more symmetrical. On standing for 7 days, material of lower sedimentation constant appeared and the system became polydisperse. This fragmentation process has been re-investigated recently in our laboratory by Mr T.-C. Tsao, not only in urea but in acid (pH 2) and alkali (pH 9–13). Although we have no information on the polydispersity of the system under these varying conditions, it is assumed, by analogy with the experiments of Snellman & Erdös, that material of high molecular weight is still present. On this basis, the sub-unit weight must be less than the number-average value of 160,000 which Tsao has obtained in alkali at pH 10·5–11. At this pH, no hydrolysis of peptide bonds occurs; at pH 13, the average particle weight falls to quite low values, but here peptide cleavage is readily detectable (Table 4). Amongst the residues with free amino groups liberated under these conditions are threonine, serine and the dicarboxylic acids.

It is not impossible that the true sub-unit weight of myosin is commensurate with that of tropomyosin, and the arguments adduced for the existence of cyclic polypeptide chains in tropomyosin are relevant for myosin also. In both cases, the results are only interpretable on these lines.

Terminal residues of other proteins. The adequacy of the author's technique in relation to that of other workers was tested on two proteins, horse globin and ovalbumin. For the former, Porter & Sanger (1948) found six valine residues/mol. of 64,000, and Desnuelle, Rovery & Bonjour (1950) only five. In ovalbumin, Desnuelle & Casal (1948) found no terminal amino groups. In the author's hands, horse globin (one determination) gave 5·5 valine residues/mol. Twice recrystallized ovalbumin (American source) was found to contain several kinds of terminal residues, amounting in all to one in 260,000 g. of protein. A sample prepared by the author, four times recrystallized, gave a mere trace of colour on the chloroform column, amounting only to one residue in 5×10^6 g. of protein. No acid-labile DNP-amino-acids could be detected.

DISCUSSION

The absence of terminal residues except in traces in tropomyosin and myosin suggests that these proteins are built of cyclic polypeptide units. By virtue of these negative findings extra care has been taken to ensure that amino-acids which give rise to the

more labile DNP derivatives are not present. The possibility of cysteine as an end group was also considered, for this acid was not investigated in Sanger's original paper. *NS* bis-DNP-cysteine was prepared and found to be as stable to acid as DNP-alanine and to run with an R value of 0·1 on a chloroform column. This acid would thus have been readily detected.

The precedent for the existence of cyclic chains in proteins is to be found in gramicidin S (Sanger, 1946), which is much simpler in structure than anything investigated here. It is not known whether ovalbumin is composed of cyclopeptides, or whether the terminal amino groups are linked to the carbohydrate residues. Such an explanation cannot hold, however, for myosin and tropomyosin in which prosthetic groups are entirely absent. It is encouraging to find that in the case of tropomyosin at least, the concept of a cyclopeptide helps to explain features which would otherwise be puzzling. These aspects are considered in the following paper (Tsao *et al.* 1951).

SUMMARY

1. Sanger's (1945) fluorodinitrobenzene method has been applied to a study of the terminal amino groups in tropomyosin, myosin and fibrinogen.

2. Tropomyosin and myosin appear to consist of cyclopeptides. It is not yet possible to decide whether the terminal residues in fibrinogen arise from the protein itself or from impurities.

3. The lysine side chains in myosin are fully reactive. There is evidence that tropomyosin contains a small proportion of unreactive lysine side chains, and the same is true of fibrin but not of fibrinogen.

4. The end-group method has been used to investigate the stability of the peptide bonds of these proteins in acid and alkaline media. Myosin at 0° is stable to alkali at pH 11 but not at pH 13; tropomyosin is stable up to pH 12.

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The Size, Shape and Aggregation of Tropomyosin Particles

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Much of the recent work on the proteins of the myofibril has been devoted to the two major components, actin and myosin. The study of the interaction of these two proteins with each other and with adenosinetriphosphate has led to the belief that this system contains the essential components of the contractile mechanism. A knowledge of the properties of the proteins concerned must be of significance in the interpretation of the process of contraction and, for this reason, we have attempted to define the structure of one of the simpler asymmetric proteins of the myofibril, tropomyosin (Bailey, 1948).

Previous investigations (Bailey, 1948; Bailey, Gutfreund & Ogston, 1948; Astbury, Reed & Spark, 1948) have suggested that tropomyosin is a proto-

type of myosin, similar in physico-chemical properties and amino-acid pattern. Like myosin, the large-angle X-ray diffraction pattern is of α-type. Tropomyosin differs from myosin mainly in its stability to denaturing agents such as ethanol, and is in fact prepared from muscle mince which has first been dried at room temperature in ethanol and ether. In salt-free solutions more alkaline than the isoelectric point, tropomyosin sols have an exceedingly high viscosity which decreases in a dramatic way as small amounts of salts are added. These observations suggested that when salts are removed, the molecules of tropomyosin aggregate to form fibrils. This conclusion was borne out by electron microscope studies (Astbury *et al.* 1948). The interesting features of this aggregation are first that it is fairly regular, giving rise to fibrils 200–300 A. wide and 3000–6000 A. long, and secondly, that it is largely electrostatic in character (Bailey, 1948).

The first estimations of the molecular weight of tropomyosin by ultracentrifugal and osmotic methods gave a value of about 90,000 for a solution of pH 6·5 and ionic strength 0·27 (Bailey *et al.* 1948). Further investigations, described in this paper, were carried out to determine the effects of concentrated urea solutions and of variations in the concentrations of salts and of hydrogen ions. Preliminary experiments (Adair, Bailey & Tsao, 1949) with solutions of ionic strengths from 0·1 to 1·1 at pH 6·5 showed that the average molecular weight varied from 135,000 to 65,000, diminishing with increasing salt concentration. It seems probable that in the most concentrated salt solutions employed, the protein molecules were still associated because a lower value for the particle size, 53,000, was obtained both in 6·7 M-urea and in 0·01 N-hydrochloric acid.

In the present paper, the changes in particle weight have been followed by the use of osmotic pressure measurements in conjunction with viscometric measurements. Experimental data obtained for the particle of molecular weight 53,000, assumed to be the monomer, show that the axial ratio is about 25. Since the average residue weight of the constituent amino-acids is known (Bailey, 1948), and assuming that three amino-acids comprise the fold (Astbury, 1941), the asymmetry can be calculated for a rigid molecule consisting of one, two or more peptide chains. The calculated value for a double-chain model agrees well with the experimental, and this structure is also suggested by the collateral chemical evidence that the monomer consists of a cyclic peptide chain (Bailey, 1951). The magnitude of the asymmetry has been confirmed by Dr G. Weber, who has applied his method of measuring the polarization of a fluorescent dye coupled to the protein (Weber, 1950). These experiments will be published separately.

MATERIALS AND METHODS

Sodium was the cation in all phosphate buffers used.

Preparation of tropomyosin. The protein was prepared from rabbit muscle by Bailey's (1948) method. Slight modifications have been introduced at stages 3 and 4 of the original preparation (see Bailey, 1951). The protein was stored at 0° as an $(NH_4)_2SO_4$ paste, or as an ethanol-ether-dried powder. In the former case, the paste was dissolved in water, dialysed in cellophan in the ice chest until free of $(NH_4)_2SO_4$, and then equilibrated against several changes of the outer liquid to be used in osmotic pressure measurements. Dry tropomyosin was dissolved in water and dialysed against 0·1 M-KCl. Any trace of turbidity at this stage indicates that impurities have not been completely removed.

Osmotic measurements. These were carried out at 0° in the toluene osmometers of Adair (1949). When the pressure head exceeded 20 cm. of toluene, the tetrachloroethylene osmometer was substituted (Adair, unpublished). Collodion membranes (Adair, 1925) were used in all experiments except those employing an alkaline medium, which caused them to become brittle and to collapse. It was found, however, that Visking cellophan tubing (Visking Corporation, Chicago, U.S.A.) withstands alkali well and has a suitable permeability. These tubes (inflated diameter $\frac{1}{2}\frac{3}{2}$ in. (1·43 cm.)) were mounted on rubber in the same way as the collodion membranes. With the latter, osmotic equilibrium was reached quite quickly (sometimes overnight), but in Visking tubes, an equilibrium was established only after several days, because of the extensibility of the material. In all cases, however, readings were taken over quite extended periods to ensure that solutions were truly equilibrated.

Measurement of protein concentration. Protein contents were generally determined by the micro-Kjeldahl method as adapted by Chibnall, Rees & Williams (1943), taking 16·7% as the N content of the protein (Bailey, 1948). For tropomyosin in concentrated urea, a known volume of solution was pipetted into a small cellophan bag, dialysed in several changes of water until free of urea, and the bag plus contents digested in the usual way. An equal volume of diffusate and an equal length of tubing were digested in a similar manner to serve as a blank and to check the thoroughness of dialysis. The analytical values were checked by refractive index measurements in the Zeiss dipping refractometer (Adair & Robinson, 1930*a*). Urea was purified according to Steinhardt (1938).

Calculation of particle weight. The extrapolation procedures of Adair & Robinson (1930*b*) were adopted. The ratio of osmotic pressure (mm. Hg) to protein concentration, i.e. P/C_v, was plotted against C_v, the latter being expressed as g. protein/100 ml. of solvent; C_v was calculated (see Adair, 1928) from the solution constants by using a value of 0·71 for the partial specific volume (Bailey *et al.* 1948) and a hydration figure of 0·248 g./g. protein, this latter determined by the method of Adair & Robinson (1931). Experiments with solutions of haemoglobin (Adair, unpublished) in equilibrium with phosphate buffers at pH 7·4 have shown that a better approximation to a straight line may be obtained by plotting $\log P/C_v$ against C_v. This method was found suitable for the observations on tropomyosin, recorded in Figs. 2 and 3*a*.

Viscosity. Measurements were made at 20° ± 0·02° in a horizontal capillary viscometer (Tsuda, 1928; Ostwald, 1933) in which the rate of flow can be varied by the use of two 15 l.

aspirators, which maintain a constant pressure during flow. The capillary had the following characteristics: radius, 0·0296 cm.; length, 29·40 cm.; flow time for 1·051 ml. of water at a pressure head of 15 cm. of water, 71·0 sec. The average rate of shear (β) was calculated from the formula of Kroepelin (1929); i.e.

$$\beta = \frac{8}{3}\frac{V}{\pi\gamma^3 t},$$

where V is the volume of liquid flowing through the capillary of radius γ in time t. At the highest shear rate employed in this investigation (1250 sec.$^{-1}$), the kinetic energy correction amounts to less than 0·4% and was neglected.

Axial ratios were assessed from the values of intrinsic viscosity by the use of Simha's equation (Simha, 1940; Mehl, Oncley & Simha, 1940), and an allowance was made for the contribution of hydration to the asymmetry factor (Oncley, 1941).

RESULTS

General considerations

In the past, a native protein in its stability zone has generally been considered to have a particle weight which is little affected by changes either in salt or protein concentration. In a few instances, the degree of polymerization is increased by an increase in protein concentration, as in the case of globin (Roche, Roche, Adair & Adair, 1932), or depends largely upon the concentration and nature of the salt present, as in the case of haemocyanin (Brohult, 1940). Outside the pH stability zone, or in concentrated urea solutions, particles may become more asymmetric, thus increasing the viscosity of the solution (Wu, 1931), and this process may be accompanied by the depolymerization of the protein into smaller units (Wu & Yang, 1932; Adair & Adair, 1938). In those cases in which depolymerization occurs, there has always been some uncertainty whether labile peptide bonds were broken; but the fact that neutral urea or guanidine solutions are as effective as acid and alkali has suggested that the native protein is often built up of several peptide chains which are generally called sub-units. This view has been fully confirmed, first by the identification of free α-amino groups in proteins (Chibnall, 1942), and more elegantly by the fluorodinitrobenzene method of end-group assay (Sanger, 1945). Moreover, there is no indication, by the application of the latter method, that new end groups are produced either in urea, or in acid at pH 2 or in alkali at pH 12 (Bailey, 1951). Two problems must thus be considered: first, the evidence for the existence of a monomer unit, its size and shape; and second, the evidence for the existence of sub-units within the monomer itself.

For convenience, the particle weight of tropomyosin in solvents which are likely to cause depolymerization will first be considered, followed by a study of aggregation phenomena in salt solutions.

Osmotic pressure of tropomyosin in urea, acid and alkaline solutions

Urea. The protein was equilibrated with a concentrated urea solution for 2 weeks before transference to the osmometers. Making use of the titration curves of Burk & Greenberg (1930) the urea solution (6·67 M) was buffered at pH 6·5 with phosphate (0·075 M, $I = 0{\cdot}1$). The value of P/C_v extrapolated to infinite dilution (Fig. 1) is 3·21, corresponding to a particle weight of 53,100.

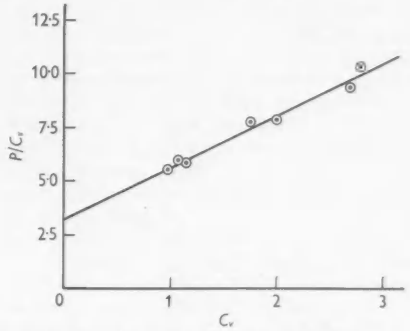

Fig. 1. Osmotic pressure of rabbit tropomyosin in concentrated urea solution at 0°. Urea, 6·67 M; phosphate, 0·075 M; pH 6·5, $I = 0{\cdot}1$. P in mm. Hg, C_v in g. protein/100 ml. solvent. $P/C_v = 2{\cdot}42C_v + 3{\cdot}21$.

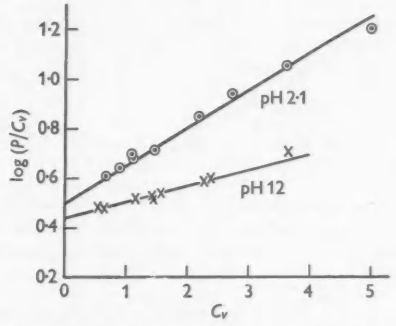

Fig. 2. Osmotic pressure of rabbit tropomyosin in acid and alkaline solutions at 0°. P and C_v as Fig. 1. Solvents: (acid) HCl, 0·01 N; KCl, 0·09 M; $I = 0{\cdot}1$, pH 2·1; (alkali) NaOH, 0·01 N; KCl, 0·2 M; $I = 2{\cdot}1$, pH 12. Acid: $\log(P/C_v) = 0{\cdot}1533C_v + 0{\cdot}5097$. Alkali: $\log(P/C_v) = 0{\cdot}06457C_v + 0{\cdot}4434$.

Acid and alkali. The equilibrating fluids used here were as follows: acid medium, 0·01 N-HCl, 0·09 M-KCl, pH 2·1, $I = 0{\cdot}1$; alkaline medium, 0·01 N-NaOH, 0·2 M-KCl, pH 12, $I = 0{\cdot}21$. The curves for both media are given in Fig. 2, in which $\log P/C_v$ is plotted against C_v. The extrapolated values of

P/C_v, 3·23 at pH 2·1 and 2·78 at pH 12, correspond to particle weights of 52,700 and 61,400 respectively.

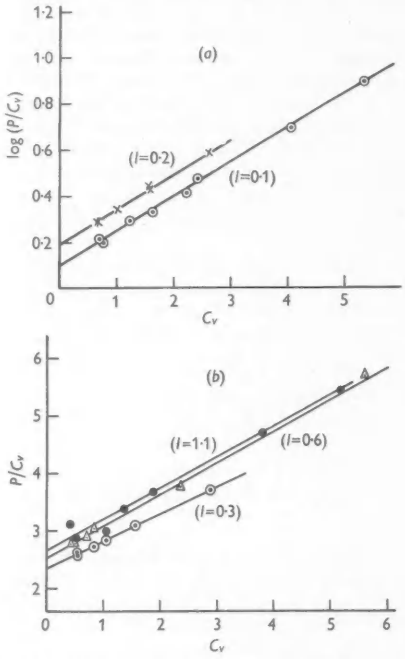

Fig. 3. Osmotic pressure of rabbit tropomyosin in sodium chloride solutions. P and C_v as fig. 1. (*a*) pH 6·5, $I=0{\cdot}1$ and 0·2. $I=0{\cdot}1$: $\log (P/C_v)=0{\cdot}150C_v+0{\cdot}100$. $I=0{\cdot}2$: $\log (P/C_v)=0{\cdot}153C_v+0{\cdot}188$. (*b*) pH 6·5, $I=0{\cdot}3$, 0·6 and 1·1 respectively. $I=0{\cdot}3$: $P/C_v=0{\cdot}467C_v+2{\cdot}36$. $I=0{\cdot}6$: $P/C_v=0{\cdot}560C_v+2{\cdot}54$. $I=1{\cdot}1$: $P/C_v=0{\cdot}544C_v+2{\cdot}64$. Detailed composition of solvents given in Table 1.

Whilst the particle weights in urea and in acid show excellent agreement, that in alkali matches the limiting value obtained in salt solutions (see next section).

Salt solutions. To determine the effect of ionic strength on the aggregation of tropomyosin, the protein was dissolved in sodium chloride solutions of varying concentration, each containing 0·06 M-phosphate buffer, pH 6·5. At a given ionic strength, the protein concentration was varied between about 0·5 and 5·5 g./100 ml. of solvent. The higher concentration represents the limit of solubility of the protein, and below the concentration of 0·5 %, the osmotic pressure is too low to be measured accurately. In solutions where aggregation is most pronounced, the logarithm of P/C_v is plotted against C_v, giving a linear relationship (Fig. 3*a*); in other cases, the conventional plot is adopted (Fig. 3*b*). The extrapolated values of P/C_v, together with the corresponding values of mean particle weight, standard deviation and osmotic coefficient are given in Table 1, which includes the data of Bailey *et al.* (1948).

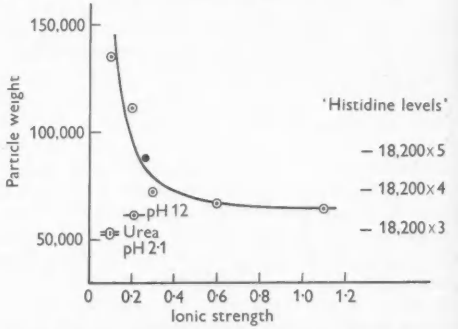

Fig. 4. Particle weight of tropomyosin in salt solutions and other media. 'Histidine levels' represent possible values of molecular weight from the histidine content (Bailey, 1948). ⊙, present work; ●, Bailey *et al.* (1948).

The aggregation of tropomyosin particles as the ionic strength is diminished is best shown graphically (Fig. 4). The most pronounced aggregation occurs in the region $I=0$–$0{\cdot}3$; in the range $I=0{\cdot}5$–$1{\cdot}1$ there is only a slight fall of particle weight, and at these

Table 1. *Particle weight of rabbit tropomyosin by osmotic pressure measurements*

Solvent*	pH	I	$(P/C_v)_{C\to 0}$	Particle weight	S.D.	ϕ† $C_v=1$	ϕ† $C_v=3$
Phosphate (0·06)	6·5	0·10	1·26	135,000	4,500	1·40	2·60
Phosphate-NaCl (0·06, 0·1)	6·5	0·20	1·54	111,000	1,900	1·43	2·88
Phosphate-KCl‡ (0·04, 0·2)	6·5	0·267	1·93	88,000	—	1·14	1·44
Phosphate-NaCl (0·06, 0·2)	6·5	0·30	2·36	72,000	600	1·17	1·51
Phosphate-NaCl (0·06, 0·5)	6·5	0·60	2·54	67,000	1,200	1·22	1·66
Phosphate-NaCl (0·03, 1·05)	6·5	1·10	2·64	64,500	3,400	1·21	1·62
Phosphate-urea (0·075, 6·67)	6·5	0·10	3·21	53,100	1,700	1·73	3·24
KCl-HCl (0·09, 0·01)	2·1	0·10	3·23	52,700	1,800	1·39	2·94
KCl-NaOH (0·2, 0·01)	12	0·21	2·78	61,400	500	1·15	1·62

* Figures in parenthesis, molarity.
† ϕ is a coefficient representing all factors which cause deviation from the simple form of the van't Hoff law.
‡ Data of Bailey *et al.* (1948).

higher salt concentrations the limiting value is virtually the same as that in alkali, but higher by about 10,000 than those in urea and acid. In salt and alkali, therefore, there would appear to be present some particles which are aggregates of those existing in a depolymerizing solvent. The reason for this is not entirely clear. Dr J. Walker has suggested (see Walker, 1949; Neuberger, 1950) that an interaction involving the carboxyl and the amidinium ion might not easily be suppressed by virtue of the formation of a stable ion pair: acids would diminish the interaction by suppressing the ionization of the carboxyl group, reagents such as urea and guanidine by competing for the amidinium ion, and alkali might be effective only at a pH which effectively suppresses the dissociation of the latter group. Such a pH would be higher than that employed here.

Viscosity

The viscous anomaly at low shear rates. The relative viscosity of tropomyosin was determined in the same solutions as those used in osmotic pressure measurements. In the case of solutions in urea, acid

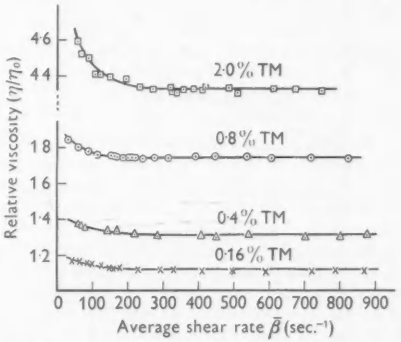

Fig. 5. Relative viscosity of rabbit-tropomyosin solutions (24·4°) at different shear rates. Solvent: KCl, 0·29 M; HCl, 0·01 N; pH 2·1. TM = tropomyosin. % signifies g. TM/100 ml. solution.

and alkali, neutral salts were added to minimize electroviscous effects. At very low rates of shear (about 300 sec.$^{-1}$) the viscosity was found to be anomalous. Typical flow behaviour is shown in Fig. 5 where relative viscosity is plotted against the average rate of shear $\bar{\beta}$. The viscosity-shear rate curves bear a superficial resemblance to those of (acto-) myosin, of tobacco mosaic virus and of other macromolecular systems. Unlike actomyosin, however, the anomaly occurs in regions of very low shear rate, and the curves thereafter are completely horizontal. In the case of actomyosin, the relative viscosity tends to fall continuously with increasing velocity gradient, and reaches a limiting value only at extremely high values of $\bar{\beta}$ (Edsall & Mehl, 1940; Portzehl, Schramm & Weber, 1950). To apply the viscosity data, it was necessary first of all to show that the origin of the anomaly was not in orientation or deformation effects, and that the limiting viscosity for the values of $\bar{\beta}$ plotted was representative of particles exhibiting Newtonian flow.

The theoretical basis of viscous anomaly has been studied from various standpoints (Philippoff, 1935, 1936, 1938; Robinson, 1939; Goodeve, 1939), which indicate that the extra disspiation of energy at low shear rates can arise in several ways. For rigid anisometric particles such as tobacco mosaic virus and mercury sulphosalicylic acid, Robinson (1939) has shown that it is due to the progressive orientation of the particles in the direction of flow. Other types of molecules show deformation during flow, e.g. poly*iso*butylene in light petroleum (Tsvetkov & Frisman, 1945) and polystyrene in *cyclo*hexane (Signer & Sadron, 1936). In these latter cases, however, the molecules examined are long, flexible chains which show the effect only at very high velocity gradients (6,000–30,000 sec.$^{-1}$). The crystallinity of tropomyosin in the intramolecular sense does not favour the idea of a flexible molecule, nor is its asymmetry on any consideration as great as in the cases mentioned; moreover, the anomaly, if due to orientation or to flexibility, should appear at high rather than low shear rates. The following experimental considerations suggest that these factors are not involved:

(1) A solution of tropomyosin in salt-free medium shows flow birefringence between crossed polaroids, whereas in all salt-containing solutions examined viscometrically, whether concentrated with respect to protein or not, no effect was observable even when the shear rates were pushed beyond 2000 sec.$^{-1}$.

(2) The magnitude of the flow anomaly decreases slightly with dilution as can be seen from Fig. 5, and for a given concentration of protein, the variation in the magnitude of the anomaly from one series to another is not pronounced. The relative viscosity of the solutions, moreover, has a slightly negative temperature coefficient: a 2% (w/v) solution in solvent of pH 2·1 and $I = 0{\cdot}3$ has a viscosity of 4·42 at 17·2° and 4·32 at 24·4° relative to the solvent at these temperatures. On the assumption that there is no change in hydration with this small increase in temperature, this last observation is directly opposed to the anomaly caused by orientation, for which a positive temperature coefficient of viscosity exists (Robinson, 1939).

(3) It is of interest to note that whereas the flow of the solvent media is perfectly Newtonian down to the smallest rate of shear investigated (approx. 30 sec.$^{-1}$), concentrated urea and guanidine hydrochloride solutions show anomalous viscosity at low shear rates. In Fig. 6, these flow curves are compared

with those of water and of solutions of tropomyosin, one in urea and one in acid. The values for water and buffer were calculated relative to their respective Newtonian levels, which were taken as unity.

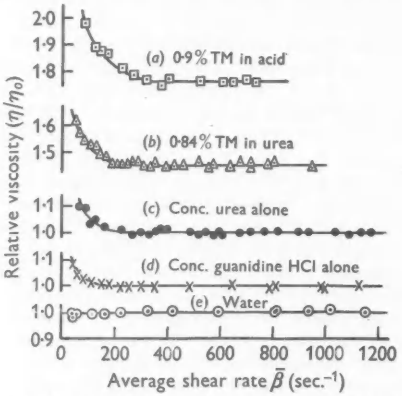
Relative viscosity (η/η_0)
(a) 0·9% TM in acid
(b) 0·84% TM in urea
(c) Conc. urea alone
(d) Conc. guanidine HCl alone
(e) Water
Average shear rate $\bar{\beta}$ (sec.$^{-1}$)

Fig. 6. Relative viscosity of various media (20·0°) at different shear rates. (*a*) KCl, 0·29 M; HCl, 0·01 N; pH 2·1; (*b*) urea, 6·67 M; phosphate, 0·075 M; pH 6·5; (*c*) as for (*b*); (*d*) guanidine HCl, 5·68 M; phosphate, 0·05 M; pH 5·4. TM = tropomyosin.

Anomalous viscosity is usually a property of colloidal solutions and not of crystalloids. (The anomaly found in strong lithium chloride solutions by Scott Blair & Schofield (1931) was later disproved by the same authors (1934).) The effect has been reported—though at high velocity gradients—in pure castor oil (Neale, 1937) and is supposed to be due to molecular association. It is conceivable that urea and guanidine molecules associate to some extent at high solute concentration, presumably by hydrogen bonding, and the anomaly in these systems is of the same magnitude as that of the tropomyosin solutions. This may be relevant with respect to the nature of the anomaly, though at the present stage of theoretical development, it is difficult to pursue the analogy further.

(4) Dr G. Weber has kindly determined the two relaxation times of tropomyosin in phosphate-sodium chloride buffer, pH 6·5 and $I = 0{\cdot}6$ by the method of polarization of fluorescent dyes (Weber, 1950). Assuming an ellipsoid of revolution, these are 8×10^{-8} and $0{\cdot}5 \times 10^{-8}$ sec. respectively, from which the rotational diffusion constants are calculated to be $H_b = H_c = 6{\cdot}3 \times 10^6$ and $H_a = 1{\cdot}9 \times 10^8$ sec.$^{-1}$, where H_b and H_c are rotational constants about the short axes and H_a about the long axis. The ratio $\bar{\beta}/H_b$ at the maximum shear rate of 1250 sec.$^{-1}$ is $\ll 1$, and is small enough to indicate that the rotational Brownian movement of the particles is sufficiently intense to oppose orientation by flow (cf. Boeder, 1932; quoted by Robinson, 1939).

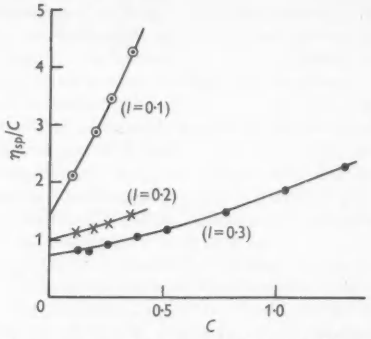

Fig. 7. Viscosity of rabbit tropomyosin in solutions at 20°. *C* in g./100 ml. solution. ⊙—⊙, in phosphate, 0·06 M; $I = 0{\cdot}1$, pH 6·5. ×—×, in phosphate, 0·06 M; NaCl, 0·1 M; $I = 0{\cdot}2$, pH 6·5. ●—●, in phosphate, 0·06 M; NaCl, 0·2 M, $I = 0{\cdot}3$, pH 6·5.

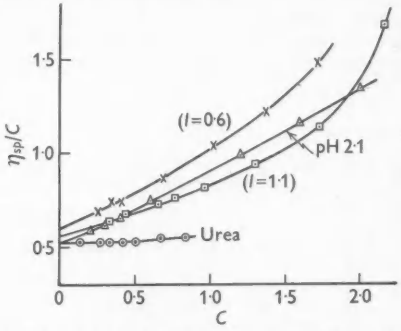

Fig. 8. Viscosity of rabbit tropomyosin in solutions at 20°. *C* in g./100 ml. solution. ×—×, in phosphate, 0·06 M; NaCl, 0·5 M; $I = 0{\cdot}6$, pH 6·5. ⊡—⊡, in phosphate, 0·06 M; NaCl, 1·0 M; $I = 1{\cdot}1$, pH 6·5. △—△, in HCl, 0·01 N; KCl, 0·29 M; $I = 0{\cdot}3$, pH 2·1. ⊙—⊙, in urea 6·67 M; phosphate, 0·075 M; KCl, 0·2 M; $I = 0{\cdot}3$, pH 6·5.

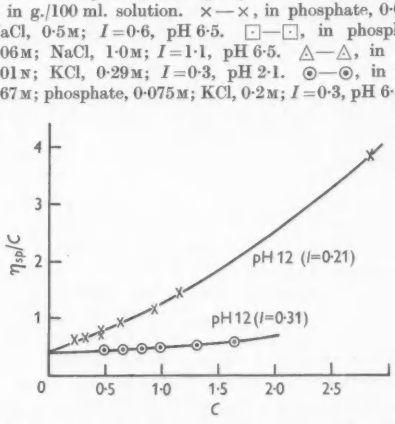

Fig. 9. Viscosity of rabbit tropomyosin in solutions at 20°. *C* in g. protein/100 ml. solution. ×—×, in NaOH, 0·01 N; KCl, 0·2 M; $I = 0{\cdot}21$, pH 12. ⊙—⊙, in NaOH, 0·01 N; KCl, 0·3 M; $I = 0{\cdot}31$, pH 12.

Taken in all, the anomalous viscosity which has been observed in the present study seems due to factors other than orientation or deformation, and the constant relative viscosity for values of $\bar{\beta}$ above 300 sec.$^{-1}$ is taken as a region in which flow behaviour is perfectly Newtonian.

The asymmetry of tropomyosin from intrinsic viscosity. The relative viscosity of tropomyosin, (*a*) in various solvents and (*b*) at varying concentrations of protein, was derived from the horizontal portion of the curves discussed above. The ratio of specific viscosity, η_{sp} (defined in Table 2), to concentration was plotted against C giving rise to sets of curves shown in Figs. 7–9 (C is here defined as g. protein/100 ml. solution). In Figs. 7 and 8 it is seen that the intrinsic viscosity falls as I increases, indicating a corresponding diminution in asymmetry as the particle weight falls. In Figs. 8 and 9 it is shown that the intrinsic viscosity is the same in acid as in urea, the two media in which the particle weight is also the same. However, particle weight and intrinsic viscosity do not follow each other in parallel fashion throughout the series; in alkali, where the former is higher than in urea and acid, the asymmetry is considerably less. Fig. 9 also shows that the dependence of viscosity upon salt concentration is very great, yet the curves extrapolate to the same value at infinite dilution.

It must be admitted that electroviscous effects may come into play in the more dilute salt solutions investigated, and the higher intrinsic viscosity obtained by extrapolation would arise from a combination of this factor and polymerization. The extent of the electroviscous contribution will alter only the absolute magnitude and not the qualitative trend of changes in asymmetry during aggregation, since evidence for polymerization is given both by osmotic pressure measurements and the electron microscope.

The calculated axial ratios derived from the viscosity increment ν by Simha's equation are given in Table 2, together with these values corrected for the estimated hydration of 25%. In the salt series there is a fall of axial ratio with increase of ionic strength, and the limiting value is very close to that in urea and in acid. Although the Simha equation cannot legitimately be employed for those solutions where there is more than one molecular species, nevertheless, the pronounced changes in the apparent axial ratio in the salt series runs parallel with the diminution of the particle weight, and can only be interpreted on the assumption that aggregation is initially an end to end and not a side to side process. Moreover, the identical values of asymmetry and of particle weight in urea and in acid lend great support to the view that in these media the protein exists in its monomeric state.

The calculation of asymmetry from X-ray data. The calculations previously applied to tropomyosin of supposed molecular weight 90,000 (Bailey *et al.* 1948) are now re-examined in the light of the newer data. From the average amino-acid residue weight of 116·4, the number of amino-acids in a molecule of the probable molecular weight 52,900 is 455. Since the intramolecular configuration is of α-type, and assuming three amino-acid residues in the fold, a single polypeptide chain would have a length of $455 \times 5{\cdot}11/3 = 775$ A. Independent chemical evidence (Bailey, 1951) suggests that tropomyosin possesses no terminal amino group, and consists of a cyclic peptide chain. The configurations that can be envisaged for such a structure are either a double polypeptide chain, or such a chain folded further to give effectively some multiple of two chains aligned side by side. A double chain can possess two configurations, one in which the peptide chains are linked through the side chains to give a grid fragment

Table 2. *Shape of rabbit-tropomyosin particles in solution*

Medium				Axial ratio				
				Simha equation		Calc. from particle wt., analysis and X-ray		
							Hydrated 25%	
pH	Solvent and ionic strength	Intrinsic viscosity* ($[\eta]$)	Viscosity increment* (ν)	Unhydrated	Hydrated 25%	Unhydrated	Ellipsoid	Cylinder
6·5	Salt (0·1)	1·40	197	53	45	67	54	58
6·5	Salt (0·2)	1·00	141	44	37	55	45	47
6·5	Salt (0·3)	0·70	99	35	30	39	32	33
6·5	Salt (0·6)	0·59	83	32	27	33	27	29
6·5	Salt (1·1)	0·57	80	31	26	32	26	28
6·5	Urea (0·3)	0·523	74	30	25	26	22	23
2·1	HCl (0·3)	0·523	74	30	25	26	22	23
12	NaOH (0·2)	0·39	55	25	21	30	25	26
12	NaOH (0·3)	0·39	55	25	21	—	—	—

* $[\eta] = \lim_{c \to 0} \eta_{sp}/C$ and $\nu = \dfrac{[\eta] \times 100}{\bar{V}}$, where C = g./100 ml. of solution, η_{sp} is $(\eta_r - 1)$, η_r being the relative viscosity η/η_o, and $\bar{V}$ is the partial specific volume.

20 A. in the plane of the side chains and 9·5A. perpendicular to them; or one in which the aggregation is effected through the backbone, giving corresponding dimensions of 10 and 19A. respectively. The overall dimensions of the two models are thus virtually identical. The mean axial ratio would thus be 775/2 (the length) divided by the mean width of 14·5A., giving a value of 26 for the unhydrated molecule.

Although tropomyosin crystals contain as much as 90% of water, the low hydration figure of 25% determined by the method of Adair & Robinson (1931) probably represents the water bound by the molecules in solution, and held on the surface. If this is the case, it can be calculated that the thickness of the hydration layer is 1·7A. if the rod is a prolate ellipsoid, and 1·1–1·2A. if cylindrical. The axial ratio would then be reduced from 26 to 22 and 23 respectively.

Owing to the many assumptions involved, and the fact that viscosity for mixtures measures the weight average and osmotic pressure the number average, it would be unwise to assume that the good agreement between these calculated values of axial ratio (about 23) and that deduced from viscosity (about 25) has no fortuitous element. But it does help to eliminate other configurations. The calculated axial ratio for a single chain would be roughly 78, for a triple chain (which seems unlikely on the chemical evidence) 13, and for a quadruple chain even less. Taken together, the data resolve the structure of tropomyosin as a double chain in the α-configuration, and the one remaining ambiguity is whether the linkage is through the side chains or the backbone.

The case of tropomyosin in alkali is exceptional, for, although the osmotic pressure measurements indicate that the average particle weight is higher than the minimum, the viscosity data suggest that the asymmetry is less than that of the monomer or of its aggregates. The molecules may assume a slightly kinked or looped configuration. Whatever changes do occur are necessarily reversible, since the protein can be recovered in the native form and recrystallized (see next section).

The stability of tropomyosin with respect to pH *and solvents*

The equilibration of tropomyosin solutions in osmotic experiments lasted for periods extending from a few days to a few weeks. Both before and after the experiment, samples were examined by electrophoresis, and in the case of exposure to solutions of high or low pH, a check was made that no peptide bonds had been split (Bailey, 1951). General properties such as solubility and crystallizability were also tested.

Solutions of tropomyosin in salt, alkali and acid. There was no evidence that tropomyosin recovered from any of these solvents differed either in solubility or viscosity from the original specimen. It was surprising to find, moreover, that the amide N, as determined by the method of Bailey (1937), was stable to alkali at pH 12 over a period of 2 weeks at 0°. In all cases, the recovered protein could be crystallized, and no breakdown could be detected by end-group assay. The phenomena of aggregation and disaggregation are thus freely reversible, and do not depend in any way upon secondary chemical changes.

Solutions of tropomyosin in urea. The intrinsic viscosity and particle weight of tropomyosin are similar both in acid and in strong urea, yet in the latter solvent the protein is irreversibly changed and can no longer be crystallized. It is still freely soluble in water, the solutions being more viscous and showing a more pronounced flow birefringence than solutions of the native protein. The increased fibrosity of the aggregates was confirmed by examining the fibrils in the electron microscope, which revealed filaments of enormous length (2–6 μ) and quite regular width. These experiments were done in collaboration with Prof. W. T. Astbury and Dr R. Reed. Prof. Astbury also found that there is no recognizable change in the intramolecular pattern after urea treatment, and if the changes induced are slight, as they appear to be, it is difficult to understand why the ability to crystallize is lost.

DISCUSSION

The present study adds little to our knowledge of the exact role of tropomyosin in muscle; the emphasis is placed upon features of structure and behaviour which seem to have some significance in problems of the biogenesis of protein fibres. It must be admitted, of course, that the aggregation phenomena are largely electrostatic in origin, and thus impermanent, but it is possible that the initial steps in the condensation of protein monomers to give true fibres arises in a somewhat comparable fashion. If the interpretation of the data is correct, there are indications that some part of the interaction persists at salt concentrations high enough to suppress the purely electrostatic attractions, for while the particle weight is at a minimum in urea and in acid, it reaches an asymptotic value some 20% higher in neutral salt solutions of quite high ionic strength. The effect is closely similar to the action of salts on the fragmentation of *Helix* haemocyanin into half molecules, where the extent of depolymerization becomes limiting at a value of 75% as the ionic strength of the medium is increased (Brohult, 1940, 1947).

The disaggregation in salt solutions has a bearing upon the state of tropomyosin in muscle. It will be recalled that the protein is soluble in water after

extraction, but is only given up by the fibres when these are immersed in M-salt solutions. It is probable that the effect of salt is to produce a depolymerization of tropomyosin ordinarily present in an aggregated condition.

The asymmetry of the monomer as deduced from viscosity data is in excellent agreement with the value calculated for a double chain in the α-configuration. As the molecules aggregate to give a variety of polymeric forms, the Simha equation cannot legitimately be applied, but for reasons given earlier (p. 33) it does appear that aggregation is end to end in the initial stages. The former calculations of tropomyosin of particle weight 90,000 (Astbury *et al.* 1948) in solutions of ionic strength 0·27 showed that the calculated dimensions for a double chain agreed only approximately with diffusion data, and even less with viscosity. (The viscosity increments used then were tentative values, lower than those reported here.) Nevertheless, the data sufficed to indicate that only a double chain was feasible.

The findings reported in the accompanying paper (Bailey, 1951) seem to be of importance in interpreting some of the unusual features of the stability of tropomyosin. It is shown there that tropomyosin contains no terminal amino group, and must thus be regarded, in the absence of detectable prosthetic group, as a cyclopeptide. This finding lends great weight, of course, to the conclusions made about the exact configuration of tropomyosin, but it also helps to explain why the ordinary denaturing processes have such little effect. If the molecule is restrained from opening out into a single chain, there is presumably little tendency for acid- and alkali-treated molecules to assume more random configurations, as do the majority of proteins. Even so, it is difficult to understand why some degree of randomness is not introduced into parts of the molecule. This might indeed be found if we had more sensitive means of detection.

The idea that still more asymmetric proteins such as myosin are built from units similar to tropomyosin has already been discussed (Bailey, 1948) and need only be amplified a little. Current work (Tsao, 1950) undoubtedly indicates that the particle which is considered to be the native myosin molecule (see Weber, 1950) can be depolymerized and, once again, no terminal amino-acids can be detected. How far this general plan extended to other fibrous proteins is a matter of very great interest, and it may be significant that the end groups found in wool keratin (Middlebrook, 1949) are of great variety and quantitatively rather meagre, more consistent with the view that they arise from non-keratinous cell inclusions.

SUMMARY

1. Rabbit tropomyosin has been studied by osmotic pressure and viscometric methods under varying conditions.

2. In salt solutions (pH 6·5) the average particle weight falls as the ionic strength of the solute increases, and approaches the value found in a depolymerizing solvent such as 6·7 M-urea. The particle weight in acid (pH 2) is the same as in urea, i.e. 53,000; in alkali (pH 12) it is somewhat higher (61,000).

3. Tropomyosin recovered from salt solutions, acid or alkali behaves in every way like the original material and can be crystallized. This is true even when the protein is stored at pH 2 or 12 for several weeks at 0°. End-group assay (Bailey, 1951) shows that no peptide bonds are split.

4. Tropomyosin recovered from urea no longer crystallizes, and after removal of urea will aggregate into fibrils of indefinite length.

5. At extremely low shear rates, the viscosity of tropomyosin is anomalous. This is due, not to orientation, but to structural factors, which are evident also in strong protein-free solutions of urea and of guanidine hydrochloride. The intrinsic viscosity of tropomyosin has been obtained from values of relative viscosity determined at moderate shear rates under conditions of Newtonian flow. The asymmetry of the particles in various solvents has been calculated from viscosity data by means of the Simha equation.

6. In its monomeric form, tropomyosin has a particle weight of 53,000 and consists of a cyclic peptide chain. The calculated asymmetry for a fully extended loop in the α-configuration agrees with that obtained from viscosity data.

7. The approximate dimensions of the monomer are as follows: length 385 A., mean width 14·5 A., axial ratio 25. Whether the double chain is held together through the side chains or the backbone linkage is unknown.

We should like to thank Prof. W. T. Astbury, F.R.S., for his continued help and criticisms and for the financial aid from his Rockefeller grant to one of us (T.-C. T.) which made the work possible. We also wish to thank Dr G. Weber and Dr P. Johnson for the invaluable discussions we have had with them, and Dr E. C. Bate Smith for the cold-room facilities in the Low Temperature Research Station.

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Studies on the Analysis of Vitamins D

1. THE REACTION OF VITAMINS D AND RELATED SUBSTANCES WITH IODINE TRICHLORIDE

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The problem of the chemical analysis of vitamins D in natural oils and synthetic irradiation products of ergosterol and 7-dehydrocholesterol has received considerable attention over the past 15 years. Relatively little success, however, has been achieved in attempts to find a substitute for bioassays for determining vitamin D potencies, although some progress has been made with the analysis of high potency synthetic concentrates. Ewing, Kingsley, Brown & Emmett (1943) have given a very full summary of the literature dealing with the chemical analysis of vitamin D. So far, the most useful reagent has undoubtedly been the improved antimony trichloride reagent of Nield, Russell & Zimmerli (1940). Since the paper by Ewing *et al.* (1943), the main advances have been due to Sobel, Mayer & Kramer (1945) with their discovery of the glycerol dichlorohydrin reaction, and De Witt & Sullivan (1946), who used a chromatographic separation on magnesia and diatomaceous earth and a modified antimony trichloride-acetyl chloride reagent in ethylene dichloride for determining vitamin D in several products. Ewing, Powell, Brown & Emmett (1948) developed two methods for determining vitamin D in irradiated ergosterol products, one by direct estimation with the improved antimony tri-

chloride reagent of Nield *et al.* (1940) and the other, after a chromatographic separation on 'Superfiltrol', by direct measurement of extinction at 265 mμ.

The four chief problems in the accurate analysis of vitamin D in natural products are (*a*) the elimination of large quantities of interfering sterols, particularly cholesterol, (*b*) the elimination of vitamin A, (*c*) the need to measure minute amounts of vitamin D, because of its great biological potency, and (*d*) to devise a sufficiently reproducible and specific reagent. No reagent so far described is very satisfactory. Spectrophotometric measurements at 265 mμ. are of doubtful value, unless irrelevant absorption is low; the various antimony trichloride reagents must be meticulously prepared to give even moderately reliable results, and no hitherto described procedure is based on a stoicheiometric reaction.

The present work describes a new reagent which undergoes a stoicheiometric reaction with the vitamins D. Part I shows how the reaction may be used for quantitative titrations and is the basis (with other work to be described later) of methods of chemical analysis for vitamin D in natural and synthetic products. These will be described in succeeding parts.

In the course of investigations into the action of inorganic halogen compounds on vitamin D, it was found that a dilute solution of iodine trichloride in chloroform, when added to a solution of calciferol in the same solvent, gave an immediate reddish violet coloration, which disappeared on addition of excess of the reagent. The reaction occurred in the cold, and appeared to take place instantaneously. When the chloroform was replaced by a more polar solvent such as acetone, no colour appeared. It seemed, from these observations, that the reaction was a simple addition of chlorine to the unsaturated linkages of the calciferol molecule, with the liberation of free iodine.

The difficulty of estimating free iodine in the presence of iodine trichloride by chemical methods prevented an unequivocal test of this hypothesis, but a study of the reaction in the visual spectrophotometer showed that the red-violet solution gave the typical absorption spectrum of iodine, with a maximum at 518 mμ.

The reaction was obviously different from all previously described reactions of iodine monochloride or trichloride with unsaturated molecules, in all of which there is iodine addition rather than liberation.

EXPERIMENTAL

Materials and apparatus

(1) *Calciferol*, m.p. 115–116°. Supplied by Glaxo Laboratories Ltd., 'Puriss. in Nitrogen' grade. (2) *Vitamin D_3*. Supplied by Glaxo Laboratories Ltd., and also by E. I. Dupont de Nemours, U.S.A. (3) *Lumisterol*, m.p. 117–118°. Obtained, by alkaline hydrolysis, from a sample of lumisteryl dinitrobenzoate, supplied by the National Institute for Medical Research, London. Recrystallized from acetone. (4) *Tachysteryl dinitrotoluate*, m.p. 151°. Supplied by G. Merck, Darmstadt. (5) *Suprasterol II*, m.p. 110–111°. Obtained, by alkaline hydrolysis, from a sample of the dinitrotoluate supplied by G. Merck, Darmstadt. (6) *Suprasterol I*. Isolated as the allophanate from the irradiation of calciferol in ether by a mercury arc lamp. (7) *Sitosteryl acetate*. (8) *Pyrocalciferyl acetate*, m.p. 79–80°. (9) *iso*Pyrocalciferyl acetate, m.p. 110°. (10) *Ergosterol*, m.p. 158–160°. Supplied by Glaxo Laboratories Ltd. Recrystallized from methanol. (11) 7-*Dehydrocholesterol*, m.p. 147°. Obtained pure, by fractional crystallization from ether of a 60% concentrate, supplied by Glaxo Laboratories Ltd. (12) β-*Carotene*. By fractional crystallization of a pure commercial grade, supplied by British Crop Driers Ltd. (13) *Vitamin A concentrate*. Molecular distillate 1,015,000 i.u./g. supplied by British Drug Houses Ltd. (14) *Iodine trichloride*. Rechlorinated until pure. (15) *Carbon tetrachloride*. B.P. grade, washed with H_2SO_4, six times with water, dried over $MgSO_4$, and redistilled in glass apparatus.

Instruments used for colorimetric measurement were the Spekker absorptiometer, the Hilger-Nutting visual spectrophotometer, and the Hilger medium quartz ultraviolet spectrophotometer.

Preliminary qualitative studies

Several classes of substance, containing different unsaturated systems, were tested to see if they gave the ICl_3 reaction under the same conditions as calciferol (i.e. simple mixing in $CHCl_3$ solution).

Sterols. The reaction was given by calciferol, vitamin D_3, ergosterol, 7-dehydrocholesterol, lumisteryl and tachysteryl dinitrotoluates, suprasterols I and II, pyrocalciferyl acetate, and *iso*pyrocalciferyl acetate.

The reaction was not given by cholesterol or sitosteryl acetate, although the former very slowly liberated iodine on shaking with a solution of ICl_3.

Vitamin A gave a positive reaction.

β-Carotene. The reaction of ICl_3 with a dilute solution of β-carotene was found to follow an unusual course. When the reagent solution is added slowly to the carotene solution, a transient greenish-blue colour occurs at the point of mixing and disappears in less than a tenth of a second. As the reagent is added, no pink coloration is observed, but the golden solution of the β-carotene becomes progressively paler. Finally, with the addition of one drop of reagent, a threshold is reached and the whole solution very suddenly turns a deep pink, as the free iodine is liberated.

The transient greenish-blue colour is, in all probability, related to an initial reaction similar to the chromogenic reactions with carotenoids given by other inorganic halides, such as $SbCl_3$; this reaction is then followed by a second reaction involving addition of the whole ICl_3 molecule to the conjugated

system, and this in turn is followed by a third reaction involving liberation of iodine. The second-stage reaction product, formed by the addition of the whole ICl_3 molecule, appears to be highly unstable near the iodine-liberation threshold, since even sudden shaking of the solution at this point serves to liberate the iodine.

Vegetable and animal oils. Almost all natural oils and fats tested reacted to some degree to liberate iodine from ICl_3. In addition, most oils absorbed very large amounts of the reagent. The only exception found was castor oil, which absorbed large amounts of the reagent, but liberated no iodine.

Aromatic hydrocarbons, and heterocyclic bases. Very pure benzene, in dilute CCl_4 solution, liberated iodine only very slowly from ICl_3. Ordinary reagent benzene reacted positively, as did toluene and xylene. When solid ICl_3 was thrown into benzene or toluene, iodine was liberated with almost explosive violence.

Pyridine and quinoline, in solution, immediately absorbed the reagent, with the separation of yellow precipitates.

Benzene derivatives. In compounds such as aromatic phenols, ketones, etc., no reaction takes place.

Mono-ethenoid compounds. Hydrocarbons such as *cyclo*hexene absorb the reagent without iodine liberation. Styrene, which contains a side-chain double bond in conjugation with the benzene ring, reacts similarly. Non-hydrocarbon molecules, such as maleic anhydride, do not react.

Di-ethenoid compounds. Isoprene (two conjugated double bonds) absorbed ICl_3 without liberation of iodine. Methyl linoleate (two conjugated double bonds) reacted similarly.

Tri-ethenoid compounds. Methyl linolenate (three unconjugated double bonds) absorbed the reagent with no iodine liberation. Methyl elaeostearate (three conjugated double bonds) liberated iodine immediately from ICl_3, without absorbing an excess of reagent.

Preliminary quantitative studies

For quantitative work CCl_4, purified as described, was found to be the best solvent. ICl_3 in this solvent is far more stable than in $CHCl_3$. CCl_4 is also the safest solvent for preparing stable dilute solutions of provitamins and vitamins D, as Paterson & Harvey (1944) have already demonstrated for ergosterol.

First studies of the reactions were made by estimating liberated iodine on the Spekker absorptiometer, using O.B. 2 filters and cells of 10 ml. capacity and 1 cm. optical depth.

(*a*) *Stability of colour.* Solutions of calciferol in CCl_4 (15·5 mg./50 ml.) and of ICl_3 in CCl_4 (46·8 mg./50 ml.) were prepared. Equal volumes (4 ml.) of each solution were mixed in the test cell and the reading taken on the absorptiometer, matching against pure CCl_4. The cells were covered with ground-glass slides to prevent evaporation, and readings were taken at intervals. Table 1 shows that the reading is reasonably stable for at least 1 min., after which a drift occurs.

Table 1. *Stability of iodine colour, obtained by interaction of calciferol and* ICl_3, *measured on absorptiometer*

(For details see text.)

Time (min.)	Reading (*E*)
0	0·121
0·25	0·121
0·50	0·122
0·75	0·122
1·0	0·122
5·0	0·130
10·0	0·134
15·0	0·139
20·0	0·144
25·0	0·144
30·0	0·150

(*b*) *Shape of titration curve.* CCl_4 solution (1 ml.), containing 2 mg. of vitamin D_3, was titrated in the test cell with a solution of ICl_3 in CCl_4 (0·052 g./100 ml.). The procedure was to add to the 1 ml. of vitamin D_3 solution a measured volume, from a burette, of the reagent, make up to 10 ml. with pure CCl_4, and immediately read on the absorptiometer.

Table 2 shows the typical titration results obtained.

Table 2. *Vitamin* D_3 (2 *mg.*) *titrated with* ICl_3. *Total volume made up to* 10 *ml. with* CCl_4

(For details see text.)

ICl_3 solution added (ml.)	Reading (*E*)
1·0	0·055
2·0	0·114
3·0	0·180
3·5	0·207
4·0	0·235
4·5	0·263
5·0	0·289
5·5	0·292
6·0	0·281
6·5	0·293
7·0	0·271
7·5	0·202

Stepwise addition of reagent produces a peak reading which is quickly followed by a second peak very nearly identical in maximum reading, and finally there is a rapid fall-off in readings as excess reagent re-absorbs the iodine liberated. Such two-peak curves are nearly always observed when the titration is carried out using small stepwise additions of reagents.

The slope of the upward portion of the curve is independent of the original amount of vitamin D titrated.

(*c*) *Attempts to remove excess reagent.* Attempts were made, with only moderate success, to remove excess ICl_3 quantitatively from the reaction mixture without removing the already liberated iodine. Small quantities of a substance such as castor oil or *cyclo*hexene were added to the titration solution, but although these substances did not absorb free iodine from simple CCl_4 solution, in the presence of ICl_3 both the latter reagent and iodine were always absorbed together. On addition of sufficient ICl_3 to a solution of *cyclo*hexene or castor oil and iodine, complete absorption may be obtained, leaving a colourless solution. If vitamin D_3 is titrated, with the addition of a small amount of *cyclo*hexene before ICl_3 addition, both substances compete for the reagent and the maximum quantity of iodine liberated is considerably lower than that liberated by vitamin D_3 alone. If the stepwise titration is carried out, adding *cyclo*hexene after the ICl_3, liberated iodine is only absorbed beyond the end point and, in this case, the maximum quantity determined is only slightly lower than the theoretical amount. Further experiments on these lines might lead to a simpler quantitative titration method, but it was not considered necessary to investigate the matter in detail, since the end point is relatively easy to determine.

It is possible that pure vitamin D_3 could be electrometrically titrated against ICl_3 in a suitable solvent, since, for example, ICl_3 is conducting in nitrobenzene, whereas iodine is not. Electrometric titration in CCl_4 should not be impossible, using perhaps high frequency (electrodeless) titration methods.

Quantitative spectrophotometric studies

Examination of the mixture of ICl_3 and calciferol in CCl_4 solutions with the Hilger-Nutting visual spectrophotometer showed, in the presence of a slight excess of calciferol, the normal iodine absorption spectrum, with a maximum at 518 mμ.: there were no other bands to be seen in the visible region. The ultraviolet absorption of the same solution showed a strong band at 265 mμ. and a faint band at 310 mμ. The absorption spectrum of the chlorinated calciferol is unknown and may account for either or both of the latter two bands. No peak was observed at 330 mμ.: this indicates the absence of free chlorine from the reaction mixture. Pure iodine in CCl_4 was found to have a molar extinction coefficient of 1116, which figure was used throughout the following calculations. Earlier determinations by Brode (1926) gave considerably lower values: the discrepancy could not be accounted for.

(*a*) *Vitamin D_3.* The vitamin D_3 molecule contains a conjugated tri-ethenoid system. If the ICl_3 reaction is quantitative and stoicheiometric, the equation followed will be

$$\text{`}D_3\text{'} + 2ICl_3 \rightarrow \text{`}D_3\text{'}\,Cl_6 + I_2.$$

For experimental test of the equation, the ICl_3 solution used contained 91·1 mg./250 ml., and the vitamin D_3 solution 40·2 mg./50 ml. The vitamin D_3 solution (1·5 ml.), mixed with 4 ml. of the reagent, theoretically gives an ICl_3/D_3 molar ratio of 2.

Table 3. *Determination of* ICl_3:I_2:D_3 *molar ratios*

Vitamin D_3 (mg.)	ICl_3 added (mg.)	Total vol. (ml.)	$E_{518\,m\mu}$	I_2 liberated (mg.)
1·206	1·456	5·5	1·21	0·766
1·206	1·602	5·9	1·25	0·849

The vitamin D_3 solution (1·5 ml.) was placed in a test cell (2 cm. optical depth) and 4 ml. reagent added. Liberated iodine was estimated by measuring the optical density of the solution at 518 mμ. The experiment was repeated using a 10% excess of reagent. Table 3 shows the results obtained. The maximum amount of I_2 was formed when 10% excess ICl_3 was added: the molar ratio, I_2/vitamin D_3 was then

$$\frac{0{\cdot}849 \times 384}{1{\cdot}206 \times 254} = 1{\cdot}06.$$

The purity of the ICl_3 was checked by measuring its extinction at 455 mμ. at an optical depth of 4 cm. The solution was found to contain only 88% of pure ICl_3. Using this correction, at the maximum point the molar ratio ICl_3/vitamin D_3 is therefore,

$$\frac{1{\cdot}602 \times 384 \times 88}{1{\cdot}206 \times 254 \times 100} = 1{\cdot}8.$$

Although this figure is slightly low, it is in good agreement with the value expected from the equation above. The slight difference between the figure found in this experiment and the theoretical value of the molar ratio is due to the presence of some monochloride in the reagent.

It was clear, from these results, that the reaction was stoicheiometric and could be used for titration, using the maximum iodine liberated as a means of measuring the end point. Fig. 1 shows the full titration curve obtained by titrating 3 ml. of the vitamin D_3 solution with ICl_3. The technique used was to place the vitamin D_3 solution in a small conical flask, add a measured amount of reagent and mix by swirling. The absorption at 518 mμ. was then rapidly measured in the test cell. Titration was continued until the peak had been passed. Simple calculation gave the iodine liberated at each point and this was plotted against volume of reagent added. The characteristic double peak is clearly shown. Beyond the end point, the absorption falls off slowly for a considerable time, since ICl_3 itself contributes about one-third of its maximum absorption at 518 mμ.

This makes it relatively easy to determine the precise end point although, of course, immediately after the peak in Fig. 1, the measured 'iodine liberated' is made up from an 'iodine + ICl_3' component.

(*b*) *Iodine trichloride-iodine back reaction.* Excess reagent combines with liberated iodine to form ICl: if this reaction were stoicheiometric, equimolecular proportions of I_2 and ICl_3 would be necessary to form three molecules of ICl. The complete vitamin D_3-ICl_3 reaction to monochloride formation would then involve 50 % excess reagent over the amount necessary for quantitative iodine liberation.

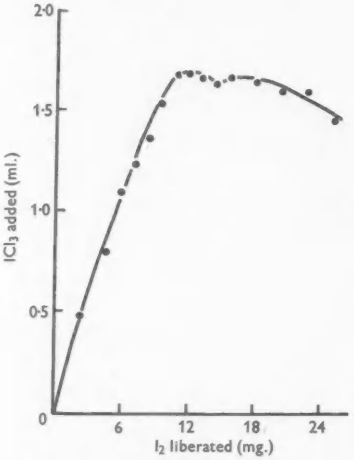

Fig. 1. Full titration curve produced by titrating 2·412 mg. of vitamin D_3 with ICl_3. Measurements carried out on visual spectrophotometer.

The vitamin D_3 solution (1·5 ml.) was mixed rapidly with 6·6 ml. of the ICl_3 solution and the mixture examined spectroscopically at 2 mm. optical depth. Only the iodine band at 518 mμ. was observed, and indeed, visually the solution still appeared to contain much free iodine. Experiment showed that several molecular proportions excess reagent were necessary to remove all the iodine, and the solution then showed the typical band at 460 mμ. due to ICl and an additional band at 330 mμ. (due to dissociation of excess ICl_3). The ICl_3-I_2 back reaction is thus not stoicheiometric: iodine is re-absorbed slowly, but eventually completely.

(*c*) *Calciferol.* The calciferol molecule contains a conjugated tri-ethenoid system in the sterol nucleus and one double bond in the sterol side chain. In most samples of calciferol examined, only the conjugated system could be titrated with ICl_3, and analytical results obtained were almost identical with those described for vitamin D_3. However, anomalous titration of all four double bonds has been found to occur with one sample of calciferol. Studies of the calciferol reaction are referred to in more detail later.

(*d*) *Vitamin A.* The reaction of vitamin A was studied, using the molecularly distilled concentrate having $1{\cdot}015 \times 10^6$ i.u./g. A solution (0·0400 g./25 ml. CCl_4) was titrated with the same ICl_3 solution, in 1 ml. quantities. Since the theoretical potency of pure vitamin A is $3{\cdot}\dot{3} \times 10^6$ i.u./g., the solution contains 0·488 mg. of vitamin A/ml. Table 4 presents the titration data.

Table 4. *Titration of vitamin A with* ICl_3 *on the visual spectrophotometer*

(1 ml. vitamin A solution taken.)

ICl_3 added (ml.)	Total vol. (ml.)	$E_{518\,m\mu}$.	I_2 liberated (mg.)
3·0	4·0	1·2	—
4·0	5·0	1·2	—
4·5	5·5	1·26	—
5·0	6·0	1·15	0·789
5·5	6·5	1·06	0·787
6·0	7·0	1·06	0·848
6·5	7·5	0·94	0·806
7·0	8·0	0·84	0·768

Calculation gives the I_2/vitamin A molar ratio at the end point as 1·71. The reaction,

$$3\text{`A'} + 10\,ICl_3 \rightarrow 5I_2,$$

requires a ratio of 1·66. In view of the impurity of the vitamin A source, the experimental result suggests that the stoicheiometric reaction is probably obeyed in the case of vitamin A.

Table 5. *Titration of β-carotene with* ICl_3

(4·5 ml. carotene solution taken.)

ICl_3 added (ml.)	Total vol. (ml.)	$E_{518\,m\mu}$.	I_2 liberated (mg.)	Remarks
0·5	5·0	3·3	Nil	Colour, golden. General absorption below 580 mμ.
1·0	5·5	1·65	Nil	General absorption below 495 mμ.
1·5	6·0	0·85	Nil	—
2·0	6·5	0·80	0·594	Colour, pink. No general absorption
2·5	7·0	0·80	0·640	—
3·0	7·5	0·76	0·653	—
3·5	8·0	0·70	0·640	—
4·0	8·5	0·68	0·661	—
5·0	9·5	0·59	0·652	—

(*e*) *β-Carotene.* The reaction of β-carotene was studied by titrating 4·5 ml. of a solution containing 0·0880 g./100 ml. CCl_4. The extraordinary reaction of β-carotene with ICl_3 has already been discussed, and is shown clearly by the spectrophotometric study, as presented in Table 5.

A study of the table shows that almost all the theoretical quantity of iodine is liberated at one point, shortly before the end point is reached: further addition of reagent then liberates normal quantities of iodine up to the end point, which is prolonged.

Calculation gives the I_2/carotene molar ratio in this experiment as 3·48. Within the limits of experimental error, therefore, the reaction is followed according to the relationship

$$3\text{'carotene'} + 22\ ICl_3 \rightarrow 11\ I_2.$$

Quantitative determinations, using the Spekker micro-absorptiometer

In the reaction between vitamins D and ICl_3, the maximum absorption reading at 518 mμ. is constant over a fairly prolonged end point. The estimation of vitamins D by titration depends on an accurate determination of the maximum, which can, under suitable conditions, be readily carried out with a Spekker absorptiometer. Determinations of absorption must, because of inherent difficulties caused by reagent instability, slight drift of readings and volatilization, be made within about a minute.

The importance of determining the smallest possible amounts of vitamin D has necessitated the use of the micro-absorptiometer for titration purposes, with the use of 0·5 ml. optical cells. However, for larger quantities of material, the Sensitive Model absorptiometer, using 5 ml. cells, may be used.

It has been found convenient to take all readings on the instrument within the range 0·075–0·250 on the density drum. Although this is a more restricted and lower range than generally used with this instrument, its use facilitates the determination of minimal amounts of material, gives satisfactory straight line standard curves, and precision of readings is usually within limits of ± 3 %.

(*a*) *Preparation of reagent.* The preparation of a stable fully chlorinated reagent is essential. As usually obtained, ICl_3 is a dry, bright-orange solid, the precise colour of which depends on its degree of purity. In air, it quickly loses chlorine to yield the dark-brown, liquid monochloride: it also loses chlorine in all solvents, at varying rates depending on the solvent. Up to 15 % decomposition of the solid reagent may occur before this state is visually noticeable by the appearance of flecks of brown liquid monochloride.

In practice the procedure adopted for obtaining a suitable reagent has been as follows. The solid (5–10 g.), ground beforehand in a mortar, is placed in a clear glass, wide-mouthed bottle, fitted with a ground glass stopper. The contents of the bottle are periodically rechlorinated by passing a stream of dry chlorine for about 10 min. or until the original bright-orange colour is regained. The bottle is kept in the dark, but, since it is of clear glass, early changes of colour in the reagent can be noticed.

(*b*) *Titration method.* The vitamin D solution is made up in CCl_4. For use on the micro-absorptiometer, the concentration should be 100–250 μg./ml. The solution is filled into a 5 ml. burette, and a 25 ml. accurately graduated burette is filled with pure CCl_4.

The reagent solution should contain about 60–80 mg. ICl_3/100 ml.; the exact strength is not critical. Dilute solutions of this kind are unstable and must be prepared immediately before titration, in either of the following ways. (i) Between 250 and 500 mg. is weighed, as rapidly as possible, directly into a few ml. of CCl_4 in a stoppered weighing bottle or small flask and dissolved in 50 ml. of CCl_4, the solid being always kept covered with solvent: some small insoluble yellow residue usually remains. A portion is diluted to working concentration. The final solution will give a reading of 0·150–0·200 on the absorptiometer when matched against pure CCl_4 in 1 cm. optical depth, using O.B.2 filters. (ii) To 10–25 ml. of CCl_4 in a clean dry beaker, a few crystals of the solid reagent are rapidly added and, after stirring gently with a glass rod, the supernatant is decanted into a second beaker when the desired concentration above the undissolved solid is reached. This may be estimated by a quick reading on the absorptiometer, or more conveniently, after a little practice, visually. Decantation may be followed by further dilution, if necessary, but the concentration should not be increased by adding further solid reagent. The main essential of either method of preparation is speed. Method (ii) is more convenient, takes about 30 sec. to complete, and is recommended for accurate titration.

The prepared solution is transferred to an accurately graduated 5 or 10 ml. burette, which should be filled only once with the reagent: if more reagent is needed, it must be prepared afresh by method (ii) or by sub-diluting the strong solution in method (i). All the burettes used must have fine-bore jets and well fitting taps: the latter should be lubricated with a grease resistant to organic solvents: an excellent preparation is the starch-glycerol paste described by Herrington & Starr (1942).

The titration is carried out in the 0·5 ml. cells of the micro-absorptiometer, which have an optical depth of 1 cm. Filters may be O.B.2 or spectrum green 604 (using, with the latter, heat-resisting filters of Calorex or Chance's ON. 19). Only one of the micro-mounts of the instrument is used, as there may be a slight variation in the dimensions of

aperture between the two mounts provided, and each cell is placed in turn in the same mount. The blank cell is filled with pure solvent. To the test cell is added first 0·05–0·20 ml. of the vitamin D solution (25–50 μg. vitamin D), then a measured volume of the reagent, and finally pure CCl_4 from the third burette to make the volume up to 0·5 ml. A small ground-glass cover slip is placed over the cell, which is inverted twice to mix the contents and then placed in the micro-mount for measurement of colour. The cell is then drained and the process repeated with increasing additions of ICl_3 to a constant volume of test solution, until the end point has been passed, as indicated by a fall in readings, and, visually, by re-absorption of liberated iodine.

It may be necessary to repeat several readings around the end point, using minimal increments of reagent in order to obtain, as accurately as possible, the true maximum reading. All readings should be made rapidly, preferably without zeroing the galvanometer each time. The cells must be quite clean, and the optical surfaces should not be touched during the titration procedure. It has been found advisable, after making up the contents of the titration cell and closing it with the glass cover slip, to wipe the optical surfaces quickly with a piece of silk fabric before inserting the cell into the instrument.

(c) *Effect of filter.* The spectrum green 604 filters give readings about 30% higher than the O.B.2 filters, leading to a corresponding increase in sensitivity. However, owing to the narrower transmission range of the former filters, the end point is much sharper and rather more difficult to determine: using the 604 filters, therefore, the reagent, near the end point, should be added in the smallest possible increments.

Titration results and standard curves

Vitamin D_3. Table 6 shows typical titration results at two levels of vitamin D_3, using spectrum green 604 filters, and Fig. 2 shows standard curves obtained by plotting maximum titration readings against micrograms of vitamin D_3, using both types of filter. The curves are linear over the range used.

Table 6. *Titration of vitamin D_3 on micro-absorptiometer*

(Total volume 0·5 ml.)

Weight of D_3 (μg.)	ICl_3 added (ml.)	Reading (E)
52·0	0·08	0·182
	0·10	0·200
	0·12	0·208
	0·14	0·215
	0·16	0·218 (peak)
	0·18	0·200
35·3	0·10	0·126
	0·12	0·132
	0·13	0·148 (peak)
	0·14	0·139
	0·16	0·134
	0·18	0·126

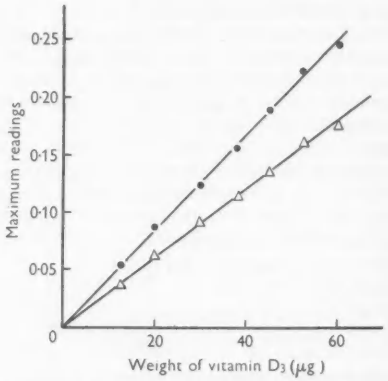

Fig. 2. Standard curves for vitamin D_3. Titration carried out with ICl_3 on the Spekker micro-absorptiometer, using two types of filter. Total volume, 0·5 ml.; optical depth, 1 cm. ●—●, spectrum green 604; △—△, O.B. 2.

Calciferol. As already stated, most samples of calciferol tested (at least twelve in number, with different origins) only three double bonds were titrated with ICl_3. With one sample, however, which was checked many times by titration on the micro-absorptiometer, a four double-bond titration was always obtained. Fig. 3 shows the two standard curves constructed by plotting maximum readings against μg. of calciferol, using O.B. 2 filters. Curve *B* corresponds to the three-bond titration and is, of course, almost identical with the vitamin D_3 curve.

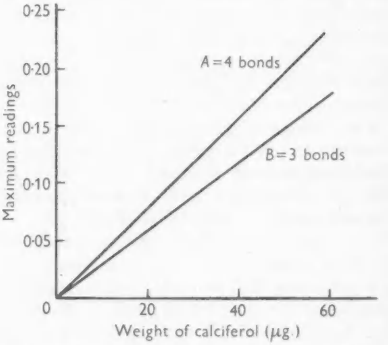

Fig. 3. Standard curves for calciferol, titrated on micro-absorptiometer; total volume, 0·5 ml.; optical depth 1 cm.; O.B.2 filters.

Table 7. *Titration of sterols on the micro-absorptiometer*

(0·5 ml. cells. Spectrum Green 604 filters.)

Substance	Weight titrated (μg.)	Maximum titration reading	No. of double bonds titrated
Ergosterol	40·0	0·170	3·05
Lumisterol	47·0	0·126	1·97
Lumisteryl dinitrobenzoate	67·0	0·136	2·10
Pyrocalciferyl acetate	58·0	0·204	2·85
*iso*Pyrocalciferyl acetate	48·0	0·183	3·10
Suprasterol II	54·0	0·151	2·05
Tachysteryl dinitrotoluate	95·0	0·145	1·73
Tachysterol	67·0	0·122	1·35

A number of experiments were carried out to determine the reason for the existence of two calciferol curves. First, chemical purification of the samples by fractional crystallization of the free sterols and their dinitrobenzoates failed to disturb the titration results. Exhaustive drying at 0·01 mm. was similarly without effect. The possibility of peroxide catalytic reaction of the fourth double bond was considered, but titration of normal samples in the presence of benzoyl peroxide or anti-oxidants such as ethyl gallate, quinol, etc. produced no change in the reactions. At present, no explanation can be offered.

Other sterols. The titration of other sterols and their pure esters was carried out as described for the vitamins D. Calculation of the number of double bonds titrated was made by computation from the standard vitamin D_3 curve. Table 7 presents the titration data and calculations for ergosterol, lumisterol, pyrocalciferyl acetate, *iso*pyrocalciferyl acetate, tachysterol, suprasterol II and 7-dehydrocholesterol.

DISCUSSION

Iodine trichloride in carbon tetrachloride solution undergoes two types of reaction with certain classes of unsaturated substances. The first type involves immediate and quantitative addition of the iodine trichloride molecule and is apparently limited to hydrocarbons and fatty acids which contain one or more isolated double bonds or two conjugated double bonds. The second-type reaction, which has been found to occur with substances containing systems of three or more conjugated double bonds and, in addition, a group of sterols related to the vitamins D, involves chlorination and liberation of iodine. Iodine monochloride, in general, reacts similarly: but, with this reagent, the second-type reaction is not so specific: cholesterol, for example, which only contains one unsaturated linkage liberates iodine from iodine monochloride. The two types of reaction are, for several reasons, remarkable, and the iodine liberation reaction is of a unique character. Although there is a wealth of literature dealing with the action of iodine chlorides on unsaturated substances, all the reactions hitherto described have involved iodination. The present reactions also differ from those previously described by taking place under the mildest possible conditions, in cold carbon tetrachloride solution. Finally, although occurring in pure non-polar solvent, the reactions are instantaneous and stoicheiometric.

Temperature is without effect either on the type, the velocity, or the quantitative character of the reaction, which proceeds identically at room temperature or at $-23°$ (the melting point of carbon tetrachloride). There is no peroxide effect involved in either type of reaction: their occurrence is independent of the presence of substances such as benzoyl peroxide or powerful antioxidants, such as quinol, pyrogallol, or ethyl gallate.

The type II reaction (iodine liberation) gives rise to speculation as to the mechanism involved. The reaction

$$R + 2ICl_3 \rightarrow RCl_6 + I_2,$$

where R is a molecule containing three conjugated double bonds, is one of semi-addition to R, or substitution of the ICl_3 molecule.

The absence of reaction between ICl_3 and cholesterol, in contrast to the reaction of ICl with the latter substance, indicates that the chlorination does not necessarily proceed through a first step of dissociation of the trichloride to the monochloride and free chlorine (although this dissociation, of course, always occurs). It is also improbable that, in carbon tetrachloride solution, sufficient ionization occurs to enter into the reaction mechanism.

It is believed that the most important indication of the reaction mechanism may be given by the observed reaction with β-carotene. The transient greenish-blue colour formed at the point of contact with the reagent is almost certainly due to an initial mesomeric change in the β-carotene molecule under the influence of an iodine trichloride molecule or a Cl^- or $[Cl_3]^-$ ion: this is akin to the type of mesomeric change (with resultant strong colour formation) produced by the action of antimony trichloride on carotenoid structures and vitamin D. The transient colour reaction of carotene with iodine trichloride is at once followed by addition of the

reagent, iodine being liberated only just before the end point. The conjugated chain is therefore almost completely saturated with iodine and chlorine atoms at first (no free chlorine is detectable spectroscopically at any point of the reaction before the end point), and the halogenated compound formed is, in the case of β-carotene, relatively stable before the iodine-liberation threshold is reached. Iodine liberation then takes place perhaps by chlorine substitution by excess reagent or by intramolecular arrangement to a more stable structure, with loss of iodine. It is possible that some sequence of events such as this, but very much quicker, occurs in the reaction with vitamins D and other reactive substances which liberate iodine, in these cases the first-formed iodine-chlorine complex being extremely unstable. This hypothesis of a two-stage reaction mechanism may be extended to cover the type I reaction. Thus, in the reaction with *cyclo*hexene, if the reacted solution is allowed to stand for 24 hr., iodine is slowly liberated: this occurs even in the absence of excess reagent and it is therefore difficult to see how the second-stage reaction, in the case of *cyclo*hexene, occurs without intermolecular rearrangement, since the products of the reaction are presumably dichlorohexahydrobenzene and iodochlorohexahydrobenzene.

It is tentatively suggested, however, that the two types of ICl_3 reaction are basically of the same character, the apparent difference being due to the extremely varying stabilities of the first-formed iodine-chlorine addition compounds. The preferential iodine-liberation reaction which takes place with systems of more than three conjugated double bonds and the series of sterols derived photochemically from ergosterol indicates that a steric factor determines these stabilities: accommodation of the large iodine atom in their molecular structures is probably too difficult for certain types of unsaturated compounds.

These hypotheses reconcile the observed reaction with theoretical requirements concerning the known polarizability of iodine chloride.

The quantitative titration of sterols on the micro-absorptiometer (Table 7) gives some interesting results. In all cases, with the exception of tachysterol, an integral number of bonds is titrated (within experimental error). Although, in the case of calciferol only three bonds usually react and the side-chain double bond is preferentially unreactive, in ergosterol, its provitamin, which contains one unconjugated and two conjugated double bonds, it is found that all three bonds react, and no anomalous behaviour has been discovered. The side-chain double bond, which is preferentially non-reactive in calciferol, is reactive in ergosterol. Even more curiously, lumisterol, which is an ergosterol diastereomer, reacts to the extent of only two double bonds, as do the acetates of pyrocalciferol and *iso*pyrocalciferol (free sterols not tested). The suprasterols have not so far been assigned a definite molecular structure: according to Müller (1935), suprasterol I contains three double bonds, but these are not conjugated. Suprasterol II also appears to have three unconjugated double bonds. Although the suprasterols are isomers of ergosterol, they do not contain the sterol skeleton. They react to the extent of two double bonds with iodine trichloride, and, therefore, if the tentative suggestions as to their structure are correct, are the only pure substances so far tested which liberate iodine from iodine trichloride and which do not possess a conjugated system.

7-Dehydrocholesterol contains two conjugated double bonds and is the only substance with less than a total of three double bonds which liberates iodine from iodine trichloride.

The titration of the purest obtainable specimen of tachysterol dinitrotoluate gave a figure of only 1·73 bonds titrated. Tachysterol itself, isolated from the dinitrotoluate by alkaline hydrolysis, was found to be very unstable, and the maximum bond titration value obtained was 1·35. Tachysterol contains, like the vitamins D, a system of three conjugated double bonds, and would be expected to show a bond titration figure of about 3. The discrepancy appears to be too large to be entirely accounted for by the known instability of tachysterol, especially in view of the very low result obtained by titration of the dinitrotoluate.

The titration of many samples of calciferol and vitamin D_3 on the micro-absorptiometer has a high degree of reproducibility, and the overall accuracy is within limits of ± 5 %. This is considered very satisfactory in view of the errors involved in reading a restricted range of the instrument drum, inherent slight drift of readings, troubles caused by volatility in the cell, the measurement of the small quantities of solutions, and inter-sample variation. The titration of a single solution of vitamin D is reproducible within limits of ± 3 %. Standard curves should be checked on at least two independent samples of vitamin D_3 and calciferol.

The limiting amounts of vitamin D that can be estimated by direct titration are governed solely by the difficulties of estimating the small amounts of iodine liberated by the reaction.

SUMMARY

1. Iodine trichloride, in carbon tetrachloride, reacts quantitatively with the vitamins D, the irradiation sterols, vitamin A, β-carotene, and some other substances, to liberate iodine. The chemistry and stoicheiometry of the reaction have been examined and discussed, and a theory of the reaction mechanism proposed.

2. A method for the quantitative estimation of the vitamins D has been developed. The determination has an overall accuracy of $\pm 5\%$.

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Studies on the Analysis of Vitamins D

2. THE ANALYTICAL PURIFICATION OF VITAMIN D BY DIFFERENTIAL SOLUBILITY, PRECIPITATION REACTIONS, AND CHROMATOGRAPHY

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Attempts to analyse synthetic irradiation products and fish-liver oils for calciferol and vitamin D_3 are fraught with unusual difficulties. These arise, in the main, from the very high biological potency of vitamin D, the wide range of potency in materials to be assayed and the low specificity of known chemical reactions. The difficulties have not been generally appreciated, and the literature shows that, with few exceptions, published methods are empirical. There have thus been few attempts to deal fundamentally with the analytical separation of the vitamins D from known interfering substances.

In fish-liver oils, the chief interfering substance is vitamin A, the removal of which is the main problem to be solved. Vitamin A may be present, weight for weight, in at least a hundred times the concentration of vitamin D, which it masks in all known reactions. Milas, Heggie & Reynolds (1941) attempted to remove vitamin A and carotenoids by preliminary treatment of concentrates with maleic anhydride in dioxan. Ewing, Kingsley, Brown & Emmett (1943) developed chromatographic methods for the elimination of vitamin A and claimed good results. Müller (1947) also described a complicated chromatographic procedure.

The analysis of vitamins D in irradiation products appears at first sight to be easier and advances have been made, particularly by Ewing, Powell, Brown & Emmett (1948). It is often assumed that most of the sterols in an irradiation product of ergosterol or 7-dehydrocholesterol do not interfere with the analysis of vitamin D by the best known colorimetric methods. Experience has shown, however, that without the best attainable purification of vitamin D by removal of sterols, such methods do not give results agreeing with biological assays, when applied to widely varying products. This is so for the method of Ewing *et al.* (1948).

In the present work, two reactions have been used to study the analytical behaviour of calciferol and vitamin D_3. Since the two substances can differ in behaviour, they have been studied independently, and conclusions drawn from study of one have not been, without test, accepted for the other. The two reactions are the antimony trichloride-acetyl chloride reaction of Nield, Russell & Zimmerli (1940) and the iodine trichloride reaction described by Green (1951).

The antimony trichloride reaction is the more specific for vitamins D, but is interfered with, to varying degrees, by the presence of cholesterol, 7-dehydrocholesterol, tachysterol (and possibly protachysterol), vitamin A and carotenoids. A disadvantage in its use is the lack of reproducibility of the reagent, which makes it difficult to obtain precise results even with fairly pure vitamin D_3. The iodine trichloride reagent is less specific, but gives a high degree of precision, and is additionally useful in analytical studies of the sterols produced by irradiation which do not react with the antimony trichloride reagent.

EXPERIMENTAL

Materials and apparatus

(1) *Sterols* (for specification, see Part 1 (Green, 1951)). (2) *Vitamin A alcohol.* Isolated by alkaline hydrolysis from pure synthetic vitamin A acetate. (3) *Light petroleum.* A.R. grade, b.p. 40–60°. (4) *Ethanol.* Pure absolute. All aqueous ethanol solutions used are recorded as v/v concentrations. (5) *Carbon tetrachloride.* Pure, redistilled from glass apparatus. (6) *Ether.* Anaesthetic. (7) *Digitonin.* Purest commercial. British Drug Houses Ltd. (8) *Floridin earth.* Prepared for chromatography by British Drug Houses Ltd. Sieved to remove particles passing 160 mesh.

All the solvents used in the spectroscopic and chromatographic studies were stringently purified and suitable for spectroscopic purposes.

The Spekker micro-absorptiometer was used throughout for colorimetric measurements. Spectrum green 604 filters were used for the ICl_3 titrations. Spectrum blue-green 603 filters were used for the $SbCl_3$ determinations in conjunction with a standard curve obtained from determinations made on pure vitamin D_3 and calciferol. Spectroscopic determinations, including some vitamin D estimations by the method of Nield *et al.* (1940) were carried out on the Uvispek spectrophotometer.

The quantitative separation of provitamins from vitamins D

The separation of the provitamins D is advisable as a preliminary step, especially with crude irradiation products of 7-dehydrocholesterol. It is also essential to remove cholesterol. Suggestions have been made, for example by Gudlet (1941), for freezing out interfering sterols from solution in methanol. Digitonin precipitation has been recommended by Raoul & Meunier (1939) and other workers, but experimental conditions for quantitative removal from vitamin D have not previously been stated.

Primary separation by differential solubility. Although ergosterol, 7-dehydrocholesterol and the vitamins D are appreciably soluble in most concentrations of aqueous ethanol above 50%, it has been found that, at a concentration of 72% (v/v) ethanol, differences in solubility are large enough to permit a primary separation of the provitamins from the vitamins D.

Solubilities of the sterols examined were determined by two methods. (*a*) By shaking an excess of the sterol with 10 ml. of 72% ethanol to give a saturated solution. (*b*) By dissolving an excess of the sterol in a known amount of pure ethanol and then bringing the concentration to 72% by the dropwise addition of water and centrifuging off precipitated material.

Amounts of dissolved sterol were measured by direct evaporation of the solution *in vacuo*, dissolving the dry residue in CCl_4, and titration on the micro-absorptiometer with ICl_3. Table 1 shows the solubilities for a number of sterols.

Table 1. *Solubilities of sterols in* 72% *ethanol*

	Solubility (mg./100 ml.)
Vitamin D_3	80
Calciferol	>100
Ergosterol	6·5
7-Dehydrocholesterol	<2·0
Cholesterol	80
Lumisterol	>100
Tachysterol	>100
Suprasterols	>100

7-Dehydrocholesterol is so insoluble in 72% ethanol that only traces could be detected with the ICl_3 reagent. Ergosterol solubility is very low, being less than a tenth of that of calciferol. Cholesterol, however, cannot be adequately separated from the vitamins D by solubility difference.

Quantitative separation by digitonin. Digitonin precipitation of sterols has been widely used for many years, but, although its use in the determination of vitamin D has been mentioned by some authors, information of a quantitative nature concerning the best procedure has been lacking. Solvents used in digitonin precipitation have been, variously, pure ethanol, 95% ethanol, acetone-ethanol, and methanol. In many methods, precipitation takes several hours to complete, which is unsatisfactory when the purpose is vitamin D analysis. Thaysen (1914) showed that precipitation in 95% ethanol is variable and often incomplete, and this was supported by Dam (1928).

Many experiments have now shown that 72% ethanol is an admirable medium for the quantitative precipitation of ergosterol, cholesterol, and 7-dehydrocholesterol digitonides, precipitation being maximal within 1 hr., and the removal of these sterols more complete than from other solvents. It is not known if the vitamins D form soluble compounds with digitonin in ethanolic solution. However, after precipitation of the provitamins from a mixture containing vitamins D, the latter substances are easily and quantitatively extracted from the filtrate by simple extraction with CCl_4. The latter appears to be a better solvent than light petroleum, which has been customarily used. If an ethanolic solution of a vitamin D, to which digitonin has been added, is evaporated to dryness, the vitamin D may also be easily extracted from the residue by washing with several small quantities of CCl_4. If, therefore, a soluble vitamin D-digitonin complex exists, it is easily split to yield unchanged the original vitamin D by simple solvent treatment.

The method which has been developed for the quantitative separation of vitamin D from ergosterol, 7-dehydrocholesterol, and cholesterol is, therefore, as follows. The sample of mixed sterols (20–50 mg.) is dissolved in 20 ml. of ethanol and 8·6 ml. of water added slowly, with stirring. The

resulting 72 % ethanol solution is allowed to settle for 5 min. and any precipitate is removed by centrifuging: the precipitate is washed with a little of the same solvent. Alternatively, the sample may be directly extracted by shaking for 10 min. with 30 ml. of 72 % ethanol in a flask fitted with a ground-glass stopper, and the solution filtered. To the filtrate obtained by either method is then added a slight excess of digitonin (about 100–150 mg. in 5 ml. 72 % ethanol) and the mixture stirred and allowed to stand for 1 hr. Precipitated material is removed by centrifuging, the clear supernatant extracted twice with 10 ml. CCl_4, and the extracts filtered and evaporated to dryness *in vacuo*. The residue is dissolved in a standard volume of CCl_4 for titration. Alternatively, the aqueous ethanol solution may be evaporated directly to dryness *in vacuo*, and the residue extracted four or five times with CCl_4 and made up to volume for titration: small losses of vitamin D, however, may occur in this latter method.

Chromatographic behaviour of vitamin D and other sterols

Vitamins D have been separated from irradiation products and fish-liver oils by the use of alumina columns. Thus Brockmann (1936) used columns of this type to separate pure vitamin D_3 from tunny liver oil by processes involving many supplementary methods of separation, and used marker dyes as vitamin D indicators. It does not appear that with alumina columns quantitative adsorption and elution have been achieved.

Three chromatographic methods have been suggested for use in vitamin D analysis. Ewing *et al.* (1943) used 'Superfiltrol' columns, but the procedure is complicated by many stages and vitamin D is determined by a difference method. Later, Ewing *et al.* (1948) employed a more satisfactory chromatographic separation of sterols and vitamin A on 'Superfiltrol'. De Witt & Sullivan (1946) claimed separation of vitamin A and sterols from vitamin D

Table 2. *Separation of vitamins D from sterols precipitable with digitonin*

Interfering sterol in mixture (mg.)			Vitamin D in mixture (mg.)	Vitamin D recovered by analysis (mg.)
Cholesterol	7-DHC*	Ergosterol		
—	—	2·002	—	0·005
—	15·000	—	5·002 D_3	4·820
—	—	20·000	2·010 D_2	2·002
—	10·020	9·982	0·505 D_3	0·498
20·050	10·010	—	0·525 D_3	0·504
—	22·000	10·010	0·625 D_3	0·648

* 7-Dehydrocholesterol.

Table 2 gives a summary of a great number of experiments. Vitamin D was determined, in synthetic mixtures containing interfering sterols, by ICl_3 titration, which reveals the presence of other sterols (except cholesterol): frequent checks, using the $SbCl_3$-acetyl chloride reagent were made and in all cases showed very good agreement with the titration method.

The method gives an absolute separation of the vitamins D from the provitamins D and cholesterol in a wide range of admixture. Vitamins D are recovered quantitatively and unchanged.

Effect of other substances on solubility separation. Other sterols do not interfere with the solubility or digitonin procedures. Small amounts of fat have little effect. Larger amounts of fat (over 50 mg.) interfere with quantitative extraction and must be removed by saponification. The solubility of β-carotene in 72 % ethanol is very low (3·8 μg./10 ml.), but may be slightly increased by the presence of other substances. Vitamin A alcohol and esters are fairly soluble in 72 % ethanol.

by zoning on a column prepared from a mixture of magnesia and diatomaceous earth.

Adsorption of vitamins D and other sterols on floridin earth. In their method for the analysis of vitamin E, Emmerie & Engels (1939) used floridin earth as an adsorbent. It has now been found that this earth is suitable for separating vitamins D from tachysterol, vitamins A, and some other interfering substances. In the present work, the chromatographic tubes used were 13 mm. diameter and about 12·5 cm. long, above the constriction. The portion of the tube below the constriction was of narrow bore to induce slow passage. The earth was prepared by boiling a thin slurry of about 5 g. with 15–20 ml. of conc. HCl for 1 min. and rapidly pouring the stirred mixture, without using suction, into the tube until a column about 7·5 cm. high was obtained. After passage of the acid, the column was washed successively with 15–20 ml. of ethanol and 25 ml. of light petroleum in small portions. During the ethanol washing, a small filter paper was placed on the top surface of the earth, and the whole

Table 3. *Adsorption and elution of sterols on floridin earth*

Ethanol concentration used for prewashing columns (%)	Sterol adsorbed (μg.)	Solvent for elution	Sterol recovered (μg.)
100	2,560 calciferol	50 ml. EtOH/ether	2,540
	95,400 calciferol	50 ml. EtOH/ether	94,800
	82,600 calciferol	100 ml. CCl_4	79,200
90	5,100 calciferol	50 ml. EtOH/ether	5,120
	4,950 calciferol	100 ml. CCl_4	4,890
	2,520 D_3	100 ml. CCl_4	2,510
	500 D_3	100 ml. CCl_4	485
	84 D_3	100 ml. CCl_4	86·5
	21 D_3	100 ml. CCl_4	20·5
	1,080 D_3	100 ml. *cyclo*hexane	260
	1,080 D_3	100 ml. benzene	1,100
	1,520 tachysterol	100 ml. CCl_4	210
	1,680 tachysterol	100 ml. CCl_4	120
85	510 D_3	100 ml. CCl_4	395

column pressed down about 1 cm. with a glass plunger.

Vitamin D is dissolved in light petroleum for the adsorption process, which is preferably carried out without suction. Quantitative adsorption always occurs, and even protracted washing with light petroleum does not result in any elution. Ergosterol, 7-dehydrocholesterol, lumisterol, tachysterol and the suprasterols are also quantitatively adsorbed on floridin. The capacity of the column is variable, as described later, but quantities of calciferol up to 100 mg. have been adsorbed on columns prewashed with absolute ethanol. In general, the capacity for mixed sterols appears very high.

Adsorption on moist columns. The adsorptive capacity of floridin is affected by its moisture content. For experimental study, columns were prepared at a standard moisture content by prewashing with aqueous ethanol, the columns then being washed with 25 ml. of light petroleum, as previously described. Columns prewashed with 90–100 % ethanol still retained a high adsorptive capacity and were capable of adsorbing up to 50 mg. of calciferol. Prewashing with 85 % ethanol severely reduced the capacity of the column, not more than about 1 mg. of calciferol or vitamin D_3 being adsorbed. Columns prewashed with 85 % ethanol are not suitable for quantitative adsorption and elution.

Adsorption in the presence of fat. Adsorption on floridin is not affected to a marked degree by the presence of small quantities of fat. Even when 0·5 g. of arachis oil is added to a light petroleum solution of vitamin D_3, none of the latter is eluted in the first 25 ml. of filtrate.

Elution of sterols. Columns prewashed with all strengths of ethanol may be completely eluted with 50 ml. of a 3 % (v/v) solution of ethanol in ether. The most useful eluting solvent, however, is CCl_4, 100 ml. of which elutes all adsorbed vitamins D from columns prewashed with 90 % ethanol and over 90 % of the adsorbed material from columns prewashed with absolute ethanol. From columns prewashed with 90 % ethanol, quantitative elution occurs with many other solvents, such as benzene and ethylene dichloride. Even as weak an eluting solvent as *cyclo*hexane slowly removes adsorbed vitamin D, on prolonged washing.

All the irradiation sterols behave similarly to the vitamins D, with the single and important exception of tachysterol. Tachysterol, adsorbed on floridin prewashed with 90 % ethanol, is eluted with CCl_4 to an extent of less than 15 %. This phenomenon does not occur when columns prewashed with absolute ethanol are used for the adsorption of tachysterol, and is probably caused by the specific destructive properties of certain types of floridin earth column towards certain structures, which are described in more detail in the section dealing with vitamin A. This difference in behaviour between the vitamins D and tachysterol, however, provides a method for their analytical separation.

Table 4. *Separation of tachysterol-calciferol mixtures on floridin earth*

Calciferol in mixture (μg.)	Tachysterol in mixture (μg.)	Calciferol recovered by elution (μg.)
890	2400	910
720	1420	760
1500	1620	1450
490	1620	475

Table 3 presents some experimental results obtained from a large number of trials of the adsorption and elution of vitamins D and other sterols. Table 4 shows the experimental separation of simple mixtures of calciferol and tachysterol. In these experiments, the mixture was adsorbed on a standard floridin earth column, prewashed with 90 % ethanol,

from light petroleum solution, the columns washed with 25 ml. of the same solvent, and then eluted with 100 ml. of CCl_4. The eluates were analysed for calciferol, after evaporation and making up in the requisite solvent.

Variations in adsorptive capacity of floridin. Different batches of floridin vary in adsorptive behaviour. Some batches have low adsorptive capacities, others adsorb sterols strongly and elution with the weaker solvents may become non-quantitative. The main differences are due to variations in particle size. It has been found advisable to remove particles which pass a 160-mesh sieve. The fraction not passing the sieve is about 50–70 % of the total and is satisfactory for chromatography. Each batch of earth, however, should be tested for (*a*) total adsorptive capacity, (*b*) non-elution of vitamins D with light petroleum, and (*c*) quantitative elution with carbon tetrachloride.

Chromatographic behaviour of vitamin A on floridin

Although Emmerie & Engels (1939) stated that floridin earth columns, 5 cm. long and 1·3 cm. in diameter, would adsorb up to 320 i.u. of vitamin A, and Awapara, Mattson, Mehl & Deuel (1946) used the adsorption of vitamin A on floridin in a method for the analysis of margarine, no study appears to have been made of the very complex behaviour of vitamin A on this adsorbent. The purpose of the present study was to devise a simple chromatographic procedure for the quantitative separation of vitamins A and D on floridin.

Effect of moisture on adsorptive capacity. The adsorption of vitamin A on floridin earth is greatly affected by the presence of moisture.

A preliminary study of this effect was carried out in the following manner. Three samples of earth were taken, one dried at 105° until moisture-free, the second (containing 3 % water) used direct from the stock bottle, and the third mixed with an added 5 % by weight of water. Each sample (1 g.) was placed in a test tube and to each was added a measured volume of a light petroleum solution (1000 i.u./ml.) of a high potency vitamin A oil. The tubes were stoppered and shaken vigorously for 1 min. Adsorption of vitamin A was indicated by the earth assuming a light-blue colour. The solutions, after filtration, were tested for unadsorbed vitamin A. Increasing quantities of vitamin A solution were added until, after adsorption, the filtrate was found to contain vitamin A. As the results in Table 5 show, the adsorptive capacity is large and is markedly reduced by the presence of water.

Table 5. *Adsorption of vitamin A on floridin earth*

Moisture content of earth (%)	Max. vitamin A adsorbed/g. of earth (i.u.)
<0·1	10,000
3	7,000
8	3,000

Vitamin A on floridin columns. The vitamin A source was a high potency fish-liver oil (140,000 i.u./g.), dissolved in light petroleum for adsorption. The prepared columns (7·5 × 1·3 cm.) after acid treatment, were prewashed with various strengths of ethanol (15–20 ml. used), followed by 25 ml. light petroleum.

Known amounts of vitamin A were adsorbed on the columns, which were then washed with 25–50 ml. light petroleum. After collection of the filtrates, elution was carried out with 3 % ethanolic ether or CCl_4. The residual vitamin A and its decomposition products were determined in the evaporated filtrates and eluates by the $SbCl_3$ reaction, and expressed as 'apparent vitamin A' recovered. Table 6 shows the recoveries and illustrates the enormous extent of vitamin A destruction which occurs under varying conditions.

The absorption of more concentrated sources of vitamin A, such as non-saponifiable extracts of fish-liver oils, molecularly distilled concentrates or pure vitamin A alcohol was investigated. In general, it was found that the more concentrated the source, the greater the yield of $SbCl_3$-reactive substance obtained in the eluates from the columns.

When a solution of vitamin A is passed through floridin earth, an intense blue colour develops at the top of the column. Depth of colour and degree of

Table 6. *Destruction of vitamin A on acid-washed floridin*

Ethanol concn. for prewash (%)	Vitamin A adsorbed (i.u.)	Apparent vitamin A in filtrate (i.u.)	Elution solvent	Apparent vitamin A in eluate (i.u.)
100	1,500	0	50 ml. EtOH/ether	100
100	3,000	0	50 ml. EtOH/ether	120
100	31,000	300	50 ml. EtOH/ether	650
100	70,000	Not determined	50 ml. EtOH/ether	1700
90	30,000	550	50 ml. EtOH/ether	280
90	70,000	650	50 ml. EtOH/ether	450
90	140,000	Not determined	50 ml. EtOH/ether	100–2000
85	70,000	1250	50 ml. EtOH/ether	90
90	3,000	Not determined	100 ml. CCl_4	80
90	10,000	Not determined	100 ml. CCl_4	150

spreading depends on three factors: (*a*) concentration of ethanol used for prewashing, (*b*) amount of vitamin A adsorbed, and (*c*) nature of vitamin A source, i.e. the presence of other saponifiable or non-saponifiable factors. On washing with light petroleum, the blue band weakens in intensity and spreads slightly, but no vitamin A is removable even on prolonged washing, except where very large amounts are put on the column, when it appears in the first 25 ml. of filtrate.

Vitamin A destruction products on floridin

Since vitamin D can be quantitatively adsorbed on and eluted from floridin earth whereas vitamin A may be quantitatively destroyed by the same procedure, the method is most valuable as a means of separating the two substances. A more detailed study of the vitamin A destruction process was made, with particular regard to obtaining eluted fractions giving the minimal interference with the estimation of vitamin D.

For these experiments, pure vitamin A alcohol, freshly prepared by alkaline hydrolysis from synthetic vitamin A acetate, was employed.

The destruction of vitamin A by acid-treated floridin results in a mixture which separates, on chromatographic analysis, into a number of products. In most experiments, the standard 7·5 cm. column was used and the amount of vitamin A used for adsorption was 7–8 mg. or about 25,000 i.u. In order to obtain additional data, columns measuring 12·5 × 2·5 cm. were occasionally used, with up to 100 mg. of vitamin A alcohol. The solvents used were all purified until a blank chromatographic procedure showed no preferential adsorption or elution of ultraviolet-absorbing impurities. The absorption data on the products of destruction were obtained by evaporation of fractions and solution of eluted material in pure ethanol, measurements being made on the Uvispek spectrophotometer, using 1 cm. cells throughout.

Columns prewashed in pure ethanol. Adsorption of 7–8 mg. of vitamin A alcohol in light petroleum on columns prewashed with pure ethanol, followed by washing with light petroleum, yields as eluate about 2 % of the original material. 100 ml. of solvent are necessary for complete elution, and the eluate, by careful fractionation, can be separated into three main substances.

The first 25 ml. of light petroleum contains a substance which shows absorption bands at 230 mμ. and 280–290 mμ., the latter peak being variable in position. In addition, the first fraction sometimes contains a small quantity of anhydro-vitamin A, with peaks at 350, 370 and 390 mμ. (the 350 mμ. band often being obscured). The second 25 ml. of light petroleum sometimes contains a little unchanged vitamin A which is adsorbed slightly more strongly than anhydro-vitamin A. Not more than 0·1 % of the original vitamin A can ever be recovered unchanged. The last fraction of the light petroleum eluate contains only a small amount of material with adsorption peaks at 240 and 290 mμ. The smallness of the total light petroleum fraction and the changes in its composition which are brought about by minute variations of chromatographic conditions combine to render the total composition of this solution non-reproducible. Anhydro-vitamin A and vitamin A, for example, are often absent. The ratio between the intensities of the 230 and 290 mμ. peaks varies considerably, perhaps indicative of the fact that two separate substances are involved. The $E^{1\%}_{1\,cm.}$ values at 230 and 290 mμ. appear to be between 150 and 300. The absorption curves of the fractions are shown in Fig. 1.

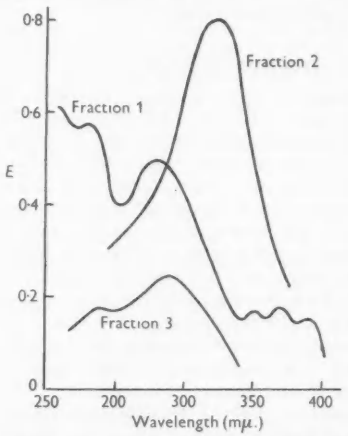

Fig. 1. Absorption curves of light petroleum filtrates produced by chromatographic destruction of 10 mg. vitamin A alcohol. All fractions in 5 ml. ethanol.

Light petroleum was usually followed by CCl_4 as eluting solvent. This solvent increases the intensity of the blue adsorption zone and diffuses it downwards to a small degree. The CCl_4 eluate is colourless and contains substances which adsorb over the whole ultraviolet range with two variable peaks, the main one at 280–290 mμ. and a subsidiary peak at 230–250 mμ. The total weight of the eluted fraction comprises about 10–15 % of the original vitamin A, and 100 ml. of CCl_4 suffices for complete elution. The $E^{1\%}_{1\,cm.}$ value of the total fraction at 290 mμ. is about 150–200. As with the light petroleum washings, careful fractionation results in a partial separation of the two absorption peaks. The substances in the CCl_4 eluate, although qualitatively similar to those found in the light petroleum washings, cannot be eluted by prolonged washing with light petroleum.

If the solvents used in the chromatographic analysis are not spectroscopically pure, selective adsorption and elution of the impurities occur on the column, and the second 25 ml. of CCl_4 may slowly elute a purple band which yields a material having a strong absorption peak at 280 mμ. This is not found to occur with pure solvents, and the substance is probably not a vitamin A decomposition product (Fig. 2).

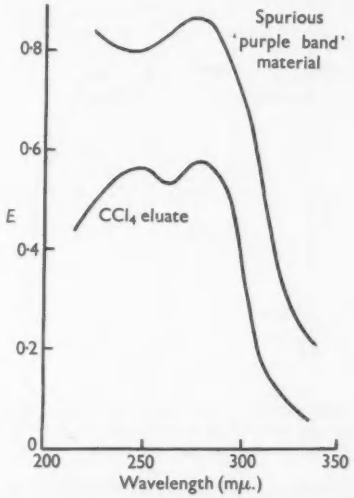

Fig. 2. Absorption curve of total CCl_4 eluate by chromatography on floridin of 10 mg. vitamin A alcohol; dissolved in 25 ml. ethanol. Absorption curve of 'purple band' material shown.

Carbon tetrachloride was followed in succession by ether, 3% ethanolic ether, and ethanol as eluting solvents. These solvents decrease the intensity of the blue band and impart to it a greenish hue. Each of the three solvents elutes a bright yellow fraction, depth of colour increasing with each fraction. The ethanol fraction is bright orange. 100–150 ml. of each solvent is sufficient to elute its own fraction. On evaporation, these coloured vitamin A decomposition products are obtained as yellow or orange solids. The ether, 3% ethanolic ether, and ethanol fractions comprise, respectively 15–20, 15–20 and 25–30% the original weight of vitamin A.

On examination of the spectroscopic curves of the three fractions, dissolved in ethanol, the substances are found to absorb over the whole ultraviolet range and up to about 470 mμ. in the visible region. All three contain three peaks, at 220–230, 280–290 and at 335 mμ. The ratio of the extinctions of the three bands in each fraction is variable, but the extinctions are of the same order. The 280–290 mμ. band is generally the strongest in all fractions, but tends to decrease in relative intensity from the ether to the ethanol fractions. However, the $E^{1\%}_{1\,cm.}$ values at the peaks decrease markedly in the fractions from ether to ethanol. The total fractions from the ether, ethanolic ether, and ethanol eluates have $E^{1\%}_{1\,cm.}$ values at 290 mμ. of approximately 800–1000, 200–350 and 70–100, respectively (Fig. 3).

Elution of the column with a more polar solvent only yields small amounts of a pale-yellow material.

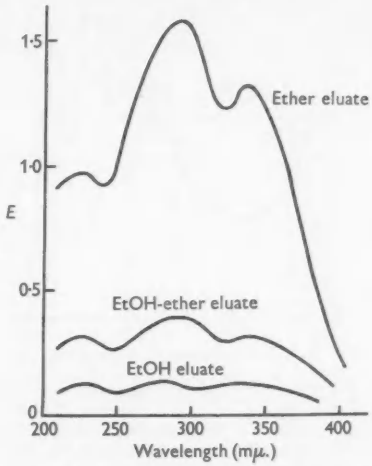

Fig. 3. Absorption curves of coloured ether, EtOH-ether, and EtOH eluates by chromatography of 10 mg. vitamin A alcohol on column washed with 100% ethanol, showing marked decrease in E values. All fractions in 100 ml. ethanol.

Columns prewashed with aqueous ethanol. Pretreatment of the floridin earth column with aqueous ethanol (in concentrations above 85%) changes the character of the vitamin A destruction process as well as decreasing the capacity of the column. In general, as the concentration of ethanol is decreased to 85%, the intensity of the blue adsorption band decreases and becomes more diffuse, while the proportion of decomposition products eluted into the light petroleum washings increases, and the proportion of the yellow and orange products eluted into the ether and ethanol fractions decreases. The substances obtained from the light petroleum fractions are, in general, similar to those obtained from ethanol-washed columns, with a great preponderance of the 230 mμ. band substance. Only traces of unchanged vitamin A are recovered. However, washing with 85% ethanol is peculiar in producing, as the main substance in the light petroleum fraction, a large amount of anhydrovitamin A, easily determined by its characteristic three peaks at 351, 371 and 392 mμ. (Fig. 4).

The CCl_4 fractions are closely similar to those obtained from ethanol-washed columns, showing mainly the 290 mμ. band. The products obtained in the ether, ethanolic ether and ethanol eluates are also similar to those obtained from ethanol-washed columns, but do not appear to be identical. In the latter three fractions, the main band at 280–290 mμ. is present, but may be displaced to shorter wavelengths. In the ethanol eluates, this band may be displaced to as low as 260 mμ. The 230 mμ. band is variable, and the 335 mμ. band is not found in products from these columns.

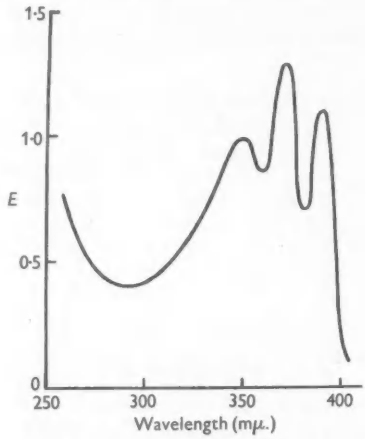

Fig. 4. Absorption curve of first light petroleum filtrate, by chromatography of 10 mg. vitamin A alcohol on column washed with 85% ethanol. Fraction dissolved in 50 ml. ethanol. Anhydro-vitamin A is the main constituent.

The effect of floridin columns containing free hydrochloric acid. All the types of columns so far described have contained no appreciable amounts of free unadsorbed hydrochloric acid. Experiments carried out to investigate the effect of columns containing free acid show that, under such conditions, the character of the vitamin A destruction process is again changed.

If the vitamin A solution is made up in light petroleum containing dry HCl gas and then chromatographed on floridin, no blue band appears on the column, and the vitamin A is quantitatively destroyed to yield a new type of product which passes completely through the column in light petroleum. It is a pale-yellow waxy solid, which is almost insoluble in ethanol, very soluble in light petroleum, and has an absorption spectrum in *cyclo*hexane showing three bands at 240–250, 280–290 and 335 mμ., all with $E^{1\%}_{1\,cm.}$ values of the order of 200 (Fig. 5).

Increasing the free HCl content still further by prewashing with ethanol containing dry HCl, preparatory to adsorbing the vitamin A, yields a substance which is slowly eluted by light petroleum as a vivid rose-pink band. However, the substance appears to be closely similar to, if not identical with, the yellow waxy solid, the absorption curve of which is shown in Fig. 5.

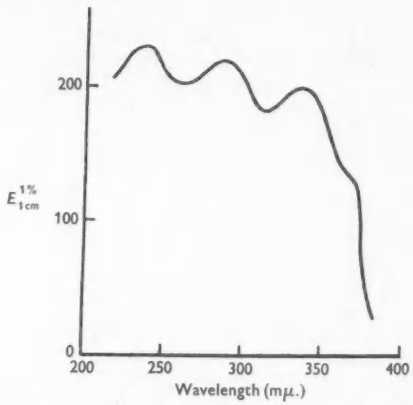

Fig. 5. Absorption curve of light petroleum filtrate containing total destruction products from vitamin A alcohol, formed on column containing free HCl.

The presence of the pronounced peak at 335 mμ. is interesting, since this peak is only found in the normal type of column eluate in the brightly coloured ether and ethanol fractions.

Vitamin D undergoes considerable destruction on free acid-containing columns, and this type of column cannot be adapted for use in the separation of vitamins A and D.

Interference of vitamin A products after chromatography in vitamin D analysis

As has already been shown, chromatography on floridin earth, under widely varying conditions, suffices to destroy quantitatively very large amounts of vitamin A. Using columns prewashed with absolute ethanol or 90% ethanol, vitamin D may be quantitatively adsorbed and eluted with either CCl_4 or 3% ethanolic ether. It is therefore possible to measure the residual interference by known amounts of vitamin A in methods of estimating vitamin D. 10 mg. (approx. 30,000 i.u.) quantities of pure vitamin A alcohol were chromatographed on floridin earth columns and residual apparent vitamin D in eluted fractions determined by ICl_3 titration and $SbCl_3$ colorimetric analysis.

Most eluted fractions containing vitamin A destruction products react to a small degree with the

$SbCl_3$ reagent. However, the method of determining vitamin D recommended by Nield *et al.* (1940) eliminates even the small residual interference due to vitamin A decomposition products in the eluates from 90% ethanol prewashed columns. By this method, which takes advantage of the fact that the absorption of the vitamin D-$SbCl_3$ colour reaction is zero at 550 mμ., the peak extinction at 500 mμ. is measured against an 'internal blank' extinction reading at 550 mμ.

Measurements on the residual interference due to vitamin A after chromatography are recorded in Table 7.

DISCUSSION

The vitamins D can be quantitatively separated from their provitamins and other precipitable sterols by differential solubility in 72% ethanol followed by digitonin precipitation. The precipitation in 72% ethanol is very rapid and is characterized by the completeness of its removal of precipitable substances. This step is particularly necessary in the analysis of vitamin D_3 concentrates obtained by irradiation of crude sterol mixtures.

Chromatography on floridin earth, under the conditions described, places on a firm basis the efficient separation of tachysterol and vitamin A from the vitamins D, being particularly valuable in that the former two substances are the main causes of gross interference encountered in vitamin D analysis. The behaviour of acid-washed floridin earth columns towards tachysterol, vitamin A, and vitamin D appears to be complex. Provided that the columns contain no free hydrochloric acid, vitamin D can always be recovered quantitatively. The capacity of the columns for vitamin D is very large and decreases as more dilute alcohol is used for prewashing, there being, however, a fairly sharp threshold between adsorption and non-adsorption at about 80% ethanol. There are no other qualitative effects.

Table 7. *Residual interference after chromatography of* 10,000 μg. *of vitamin A*

Column prewash (% ethanol)	Type of eluate	ICl_3 reaction	'Apparent vitamin D' (μg.)*	'Apparent vitamin D' (μg.)†
100	3% ethanolic ether	Strongly positive	—	—
	CCl_4	Negligible	11·0	6·0
90	3% ethanolic ether	Strongly positive	—	—
	CCl_4	Negligible	20·0	0·5
85	CCl_4	Negligible	14·0	2·5

* Maximum 'apparent D' by measuring at 500 mμ. against external blank.
† 'Apparent D' measured against internal blank at 550 mμ.

The orange-coloured decomposition products of vitamin A eluted with ether react strongly with ICl_3, behaving to this reagent like carotenoid pigments in giving a delayed reaction, accompanied by transient blue colour formation. The CCl_4 eluates react to a negligible extent with ICl_3. As measured by the $SbCl_3$-acetyl chloride reagent, using the internal blank method, the interference due to 'apparent vitamin D' is very small in all cases. The 90% ethanol-washed column is, from every point of view, the most suitable for the separation of vitamins A and D. The CCl_4 eluates from vitamin A destruction on this type of column react with the $SbCl_3$ reagent to give a product whose absorption curve over the range 500–550 mμ. is almost linear and of equal intensity: the internal blank method of measurement therefore eliminates interference almost entirely.

Table 8. *Separation of vitamin A and vitamin* D_3

Vitamin A alcohol in mixture (mg.)	Vitamin D_3 in mixture (μg.)	Recovery of vitamin D_3 (μg.)	
		ICl_3 assay	$SbCl_3$ assay
10,000	120	Not determined	112
25,000	300	280	292
19,000	505	520	485
10,000	50	Not determined	43

Table 8 shows the experimental separation of mixtures of pure vitamin A alcohol and pure vitamin D_3 by chromatographic analysis on floridin columns, prewashed with 90% ethanol and eluted with CCl_4.

Tachysterol is destroyed to a large extent by single passage through a column washed with 90% ethanol, but not if the column is washed with pure ethanol. This effect of aqueous ethanol in changing the character of adsorption is more marked with vitamin A. The complex destruction of vitamin A is conditioned by two main factors, acidity and moisture content of the column. When the vitamin A decomposition products are examined spectroscopically, a remarkable feature observed is the persistence of the position of the three main absorption bands (one of which may be missing) in all the fractions tested. This fact indicates that the destruction is probably due to a process of polymerization. A series of substances is formed, the general spectroscopic absorption of which is extended into longer wavelengths with increased

molecular weight. The basic molecular structure of the substances is probably similar.

The consistent appearance of the 280–290 mμ. band of low intensity is of some interest, since a similar low intensity band is characteristic of kitol, a dimeric polymer of vitamin A, described by Embree & Shantz (1943*a*) and was also observed in the sub-vitamin A isolated from shark-liver oil by Embree & Shantz (1943*b*). A structural relationship between these substances is indicated.

The influence of prewashing with 85 % ethanol in producing large amounts of anhydro-vitamin A from the column is unexpected, since 'cyclization' of vitamin A is usually produced by the presence of acid in anhydrous solvents. After 85 % ethanol washing, however, the columns have a high moisture content, and it is evident that there is a water-acid relationship which profoundly affects the decomposition and which, under certain conditions, leads to 'cyclization' of vitamin A.

For analytical purposes, the 90 % ethanol washed column is the most useful. By its use, with one liquid chromatogram, vitamin D can be separated from tachysterol and vitamin A. β-Carotene is destroyed on the column and does not interfere.

SUMMARY

1. Methods for the quantitative separation of vitamins D from interfering sterols and vitamin A have been developed.

2. The methods involve removal of provitamins and precipitable sterols by differential solubility and precipitation with digitonin under controlled conditions and a single chromatographic analysis on floridin earth.

3. The destruction of vitamin A on floridin earth has been studied.

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Studies on the Analysis of Vitamins D

3. EXPERIMENTS ON THE SEPARATION OF VITAMIN D FROM VITAMIN A AND OTHER SUBSTANCES BY CONDENSATION TECHNIQUES AND SELECTIVE IRRADIATION

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Proposals for the analytical separation of vitamins D from interfering substances include the method of selective condensation with maleic or citraconic anhydrides and that of removing interference by selective destruction with filtered ultraviolet radiation. The first method has been recommended for the removal of vitamin A in fish-liver oils and also for the removal of tachysterol in synthetic irradiation products. The second method has been recommended for the destruction of vitamin A.

Vitamin A, carotenoid substances in general, the vitamins D, tachysterol and some other sterols condense, under suitable conditions, with maleic and citraconic anhydrides to give inactive substances capable of easy elimination. Windaus & Lüttringhaus (1931) stated that vitamin D reacted very

slowly with maleic anhydride. Kawakami (1935) separated vitamin A from fish-liver oil concentrates by condensing acetylated material with maleic anhydride in benzene for 15 hr. Hamano (1935) used a similar method. The most important analytical proposal has been due to Milas, Heggie & Reynolds (1941), who put the method to quantitative use in a projected spectrophotometric assay for vitamin D in fish-liver oils. After removing sterols by freezing, the vitamin A-containing concentrate was condensed in dioxan (freed from peroxides) with maleic anhydride for 1 hr. On spectroscopic evidence, elimination of vitamin A was claimed, but no experimental work on the effect on pure vitamin D was described.

The removal of vitamin A in concentrates by selective irradiation has been suggested by several authors and has been used in the spectroscopic analysis of vitamin A, for example by Chevallier & Chabre (1934), Neal, Haurand & Luckmann (1941), Neal & Luckmann (1944) and Little (1944). Most workers have used unfiltered radiation, but Little showed that the effective wavelengths for vitamin A destruction were between 300 and 400 mμ., and he stated that all other wavelengths should be filtered out. He claimed selective destruction of vitamin A under these conditions. The effect of these radiations on small amounts of vitamin D does not appear to have been adequately studied.

EXPERIMENTAL AND RESULTS

Materials and methods

(1) *Vitamin A, vitamins D, and sterols.* For specification, see Parts 1 and 2 (Green, 1951*a*, *b*). (2) 1:4-*Dioxan.* Freed from peroxides by standing for 24 hr. over sodium amalgam and then distilling from the same material, collecting the fraction boiling at 99–102°. (3) *Maleic anhydride.* Prepared freshly by distillation from P_2O_5. (4) *Citraconic anhydride.* Redistilled.

Vitamin A was determined by the $SbCl_3$ colour reaction and vitamins D by the $SbCl_3$-acetyl chloride reagent of Nield, Russell & Zimmerli (1940) and the ICl_3 titration method of Green (1951*a*).

Condensation with maleic and citraconic anhydrides

The condensation method was that described by Milas *et al.* (1941). However, in addition to the maleic anhydride-dioxan condensation used by these authors, condensations were also investigated, using anaesthetic ether and benzene as media and substituting citraconic anhydride for maleic anhydride. The vitamin A or sterol-containing material was saponified where necessary, and then dissolved in 5 ml. of the condensation solvent. The condensing agent (0·2–0·5 g.) was added and the mixture heated under reflux on a water bath for 1 hr. After cooling, 15 ml. of 0·5N-ethanolic KOH and, after standing 5 min., 20 ml. of water was added. Uncondensed material was extracted several times into ether and the ethereal extract washed twice with water and dried with sodium sulphate. After evaporation of the solvent, the residue was analysed by the methods referred to above.

Vitamin A. The claims of Milas *et al.* (1941) for their method could not be substantiated. Experimental trials on the dried non-saponifiable fraction of a high potency fish-liver oil (140,000 i.u./g.) in which the dioxan-maleic anhydride condensation was used showed that the elimination of vitamin A was not complete. These results are shown in Table 1. When initial quantities of vitamin A of the order 10,000–15,000 i.u. were used, 200–400 i.u. of vitamin A were recovered. When larger amounts of starting material were used, elimination was even less satisfactory, up to 10 % of the original vitamin A

Table 1. *Condensation of vitamin A with maleic anhydride in dioxan*

(Starting material, fish-liver oil (140,000 i.u./g.) saponified.)

Original amount of vitamin A (i.u.)	Vitamin A recovered (i.u.)
10,000	200
14,000	350
140,000	7,000
200,000	16,000

Table 2. *Condensation of vitamin A in ether and benzene*

Starting material	Original amount of vitamin A (i.u.)	Solvent	Condensation agent	Vitamin A recovered (i.u.)
Fish-liver oil saponified	10,000	Benzene	Maleic anhydride	350
Fish-liver oil saponified	10,000	Ether	Maleic anhydride	700
Molecularly distilled concentrate	10,000	Benzene	Maleic anhydride	250
Molecularly distilled concentrate	10,000	Ether	Maleic anhydride	400
Molecularly distilled concentrate	10,000	Benzene	Citraconic anhydride	400
Molecularly distilled concentrate	10,000	Ether	Citraconic anhydride	550
Fish-liver oil saponified	140,000	Benzene	Maleic anhydride	1,200
Fish-liver oil saponified	140,000	Ether	Maleic anhydride	20,000
Fish-liver oil saponified	100,000	Benzene	Maleic anhydride	950

being recovered unchanged. Similar unsatisfactory results were obtained, even after removal of sterols from the vitamin A concentrate, by freezing in methanol as recommended by Milas *et al.* (1941).

A series of condensations was carried out by refluxing in ether and benzene, and the results are expressed in Table 2. Maleic and citraconic anhydrides were employed, and, in addition to the non-saponifiable fraction of a high potency fish-liver oil, a molecularly distilled concentrate of vitamin A ($1{\cdot}015 \times 10^6$ i.u./g.) was used. When the original quantity of vitamin A was 10,000–15,000 i.u., maleic anhydride gave slightly better results than citraconic anhydride, benzene being a somewhat better medium than ether. In all these experiments, over 250 i.u. of unchanged vitamin A were recovered. When over 100,000 i.u. of vitamin A were taken, benzene was clearly the best medium for reaction, elimination of vitamin A in this solvent exceeding that obtained in dioxan or ether. Condensation of these large amounts of vitamin A in ether was ineffective, 10,000–20,000 i.u. of vitamin A being regularly recovered.

Increasing the amount of condensation agent and increasing the time of reaction to 1·5 hr. had very little effect on improving the elimination of vitamin A.

Vitamin D and other sterols. Milas *et al.* (1941) described no experiments on the effect, under their analytical conditions, of the maleic anhydride-dioxan condensation on pure vitamin D. Pirlot & Rougir (1947) described a method for the analysis of calciferol in ergosterol-irradiation resins, in which they claimed, on empirical grounds, removal of tachysterol by condensation with citraconic or maleic anhydrides under reflux in benzene for 30 min. They found that, under these conditions, calciferol was stable to the condensation reagent and gave unchanged values with the $SbCl_3$-acetyl chloride reagent after treatment.

Table 3 shows the results obtained by effecting maleic anhydride condensation with pure calciferol and vitamin D_3, using the same conditions as for vitamin A. There was considerable difference in the behaviour of calciferol and vitamin D_3, the latter being more vigorously attacked by the reagent. From the reaction in ether, 85% of unchanged vitamin D_3 was recovered and over 90% of calciferol. When benzene was used as a condensation solvent, as described by Pirlot & Rougir, only about 80% of calciferol was recovered and 60–65% of vitamin D_3. Shortening the reaction time in benzene to 30 min. did not increase the recoveries. Condensation with maleic anhydride in dioxan, contrary to the empirical findings of Milas *et al.* (1941), was found to destroy large amounts of calciferol, and vitamin D_3 was destroyed almost quantitatively by this method.

Dioxan-maleic anhydride condensations were conducted on lumisterol, tachysterol, pure suprasterol II, and a sample of mixed suprasterols. Recovered sterols in the end products were determined by iodine trichloride titration. These results are also shown in Table 3. Tachysterol was always completely destroyed. Lumisterol was found to resist attack to a greater extent than calciferol, 50% of unchanged lumisterol being recovered after 75 min. reaction time. The suprasterols, which have non-conjugated structures, were recovered quantitatively after reaction times up to 2 hr.

Separation of vitamins A and D by selective irradiation

Irradiation procedure. The source of ultraviolet radiation was a Hanovia S. 500 mercury lamp. The lamp was mounted in a well ventilated metal housing, fitted with an opening 3 in. in diameter, parallel to the long axis of the lamp. Behind the lamp was fitted a curved aluminium reflector. At a distance of 15 in. from the lamp, in a vertical

Table 3. *Condensation of vitamin D and sterols*

Sterol used	Sterol weight (mg.)	Solvent used	Reaction time (min.)	Sterol recovered (mg.)
Calciferol	5·00	Ether	60	4·60
Calciferol	5·00	Benzene	60	4·10
Calciferol	2·50	Benzene	60	2·06
Vitamin D_3	3·80	Ether	60	3·25
Vitamin D_3	3·80	Benzene	60	2·55
Vitamin D_3	3·80	Benzene	30	2·50
Vitamin D_3	4·00	Dioxan	60	Nil
Vitamin D_3	8·00	Dioxan	60	Nil
Calciferol	4·80	Dioxan	45	1·86
Calciferol	5·60	Dioxan	45	2·25
Calciferol	5·80	Dioxan	75	0·59
Tachysterol	5·20	Dioxan	60	Nil
Lumisterol	5·90	Dioxan	45	3·55
Lumisterol	3·90	Dioxan	75	1·95
Suprasterol II	5·40	Dioxan	75	5·35
Mixed suprasterols	10·50	Dioxan	75	10·60

direction, was a filter rack into which filters, 3 × 3 in. (7·5 × 7·5 cm.), could be placed for the selective elimination of desired wavelengths. It was not practical to use distances shorter than 15 in. from lamp to irradiation vessel when using filtered radiation, since the heat generated by the lamp had an adverse effect on the filters. A cabinet round the lamp-house screened off all light in a vertical direction except that which passed through the filters.

The irradiation vessel was a 250 ml. flat-bottomed fused-silica flask, which, in most experiments, stood directly on the surface of the filter. During irradiation, the flask and its contents warmed up to about 50°, no cooling being used.

A study was made of the effect of filtered ultraviolet radiation on vitamin A and the vitamins D in dilute solution in an inert solvent. Each sample was irradiated in dilute CCl_4 solution for a standard time and the unchanged vitamins A or D determined, after evaporation of the solvent, by the appropriate method.

Irradiation of vitamin A. The volume of solution being irradiated was standardized at 20 ml. in all experiments. Amounts of vitamin A irradiated varied from 1000 to 5000 i.u. Each solution was irradiated until a test on a small portion showed that the vitamin A had been totally destroyed. Table 4 shows the results of irradiating vitamin A solutions under varying conditions. Two filters were used, an Ilford 828 filter, transmitting ultraviolet radiation only in the range 300–400 mμ. and a Wratten 39 filter, transmitting in the range 300–500 mμ.

No significant difference in the rates of destruction of vitamin A was found between the two filters. There was a slight increase in the rate of destruction when more concentrated solutions of vitamin A were irradiated. Rates of destruction increased as the purity of the vitamin A solution was increased. As Table 4 shows, when a molecularly distilled concentrate of vitamin A was used, shorter times were necessary for total destruction of an equivalent amount of vitamin A than with a high potency fish-liver oil; this difference is probably accounted for solely by the internal filtration effect of other substances in the oil, which absorb in the same range as vitamin A.

Selective ultraviolet irradiation was also efficacious in removing artifacts of vitamin A produced by cyclization and the destructive chromatographic adsorption on floridin earth (Green, 1951*b*). Indications were that the rates of destruction of these substances were approximately the same as the rate of destruction of vitamin A. The products after irradiation were, in all cases, inactive towards the $SbCl_3$ and the ICl_3 reagents.

Irradiation of calciferol and vitamin D_3. Solutions of calciferol and vitamin D_3 of varying concentration in CCl_4 were irradiated at a distance of 15 in. from the S. 500 lamp. Both types of filter were used.

Table 4. *Irradiation of vitamin A in* CCl_4

Vitamin A source	Amount of vitamin A irradiated (i.u.)	Filter	Time for total destruction (min.)
Fish-liver oil 140,000 i.u./g.	1000	Wratten 39	8·5
Fish-liver oil 140,000 i.u./g.	2000	Wratten 39	14·0
Fish-liver oil 140,000 i.u./g.	1000	Ilford 828	9·0
Fish-liver oil 140,000 i.u./g.	2000	Ilford 828	14·0
Fish-liver oil 140,000 i.u./g.	5000	Ilford 828	35·0
Molecularly distilled concentrate	1000	Ilford 828	5·5
Molecularly distilled concentrate	2000	Ilford 828	9·0

Residual solutions were analysed for unchanged vitamin D by the $SbCl_3$-acetyl chloride reagent. Table 5 shows the results obtained.

Table 5. *Irradiation of vitamins D*

Original wt. of vitamin D (mg.)	Filter used	Irradiation time (min.)	Vitamin D recovered (mg.)
1950 D_3	Ilford 828	30	1040
1950 D_3	Ilford 828	10	1930
1950 D_3	Wratten 39	10	1960
2400 calciferol	Wratten 39	10	2360
2400 calciferol	Wratten 39	20	2010
600 D_3	Ilford 828	10	370
350 D_3	Ilford 828	5	120

Using about 2 mg. of either calciferol or vitamin D_3, good recoveries were obtained with both types of filter when irradiation times up to 10 min. were used. Longer irradiation times resulted in partial destruction, only 53 % of vitamin D_3 being recovered after 30 min. irradiation. Poor recoveries were obtained when smaller quantites of vitamin D were used, even when the irradiation time was as short as 5 min. Preliminary analytical treatment of vitamin D-containing materials appeared to increase the destruction of vitamin D by irradiation.

DISCUSSION

The use of selective condensation with maleic or citraconic anhydrides, which has been adopted as an analytical step in the assay of irradiation products

and fish-liver oils for vitamin D, must be treated with reserve.

The experiments which have been described have shown that the treatment is ineffective in quantitatively removing vitamin A or, alternatively, in removing enough vitamin A to eliminate interference in the vitamin D determination. The procedure of Milas *et al.* (1941) completely destroys large amounts of vitamin D_3 and over 60 % of calciferol, even when large quantities of the latter are used. The safest solvent in which to carry out the condensation is ether, but unfortunately, although good recoveries of calciferol and vitamin D_3 have been obtained with this solvent, complete elimination of vitamin A is not possible within reasonable times of reaction. Condensation in benzene, as used by Pirlot & Rougir (1947), results in up to 20 % loss of calciferol and about 40 % loss of vitamin D_3.

The method of selective ultraviolet irradiation to remove vitamin A from vitamin D does not appear to be suitable for routine use. It is possible that, under carefully controlled conditions, small amounts of vitamin A (the bulk of which has been removed by other methods) can be successfully eliminated from vitamin D-containing material. Experiments have shown that although vitamin A can be smoothly destroyed by filtered radiation in the 300–400 mμ. range, the irradiation, under similar conditions, of small amounts of vitamin D results in inevitable losses.

SUMMARY

1. The analytical purification of the vitamins D by the methods of maleic and citraconic anhydride condensation and by selective ultraviolet irradiation has been investigated.

2. The condensation method is not suitable for quantitative removal of vitamin A, and losses of vitamins D always occur in its use. Vitamin D destruction by the maleic anhydride-dioxan method is particularly severe.

3. Selective ultraviolet irradiation successfully destroys vitamin A, but the method cannot at present be used to separate vitamin A from vitamin D.

4. Methods for the analysis of vitamin D which include either of the above methods of purification are of doubtful value.

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Ester Formation by Yeasts

1. ETHYL ACETATE FORMATION BY *HANSENULA* SPECIES

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The ability to form traces of esters is a property of many yeasts and is partly responsible for the bouquet of wines; however, comparatively few species are able to form esters in considerable amounts. Prominent amongst these are the two species of *Hansenula*, *H. anomala* and *H. saturnus*. Very little in the way of quantitative work appears to have been done on ester formation by yeasts. Gomes (1945), in a study of the microflora of the Douro valley, reported that a strain of *H. anomala* var. *sphaerica* (Naegeli) Dekker produced 9·59 g. total esters (as ethyl acetate) from 145 g. of total reducing sugars and at the same time ethanol formation was diminished. Gray (1949) isolated derivatives of acetaldehyde, ethanol and acetic acid from the products of the fermentation of glucose by *H. anomala* (Hansen)

Sydow, but his evidence that the ester formed by this organism is ethyl acetate is not conclusive. From the products of acid hydrolysis of the neutral fraction distilling at 55–70° he isolated crystalline derivatives of ethanol and acetic acid. The neutral distillate must undoubtedly have contained some free ethanol and therefore the isolation of ethanol derivatives does not prove conclusively that the ester was ethyl acetate. A study of the respiratory processes and dehydrogenases, particularly the ethanol dehydrogenase, of *H. anomala* was made by Usami (1942), but did not include any work on ester formation.

It was considered possible that *Hansenula* species might afford suitable material for studying the mechanism of ester formation in biological systems and the factors associated therewith. This paper deals with the qualitative and quantitative analysis of the products of the metabolism of glucose by *H. anomala* and *H. saturnus*.

EXPERIMENTAL

Organism

The strains of *H. anomala* and *H. saturnus* used in this work were obtained from the Carlsberg Laboratory, Copenhagen, through the kindness of Prof. O. Winge, and are nos. 119 and 56 respectively in the Carlsberg collection of yeasts.

Growth

The growth medium contained 4% (w/v) glucose (sterilized separately), 0·1% $NH_4H_2PO_4$, 0·1% $(NH_4)_2HPO_4$, 0·2% KH_2PO_4, 0·2% Difco yeast extract and 10 ml./l. of a trace-element solution containing 2% (w/v) $MgSO_4.7H_2O$, 0·1% NaCl, 0·05% $FeSO_4.7H_2O$, 0·05% $ZnSO_4.7H_2O$, 0·05% $MnSO_4.3H_2O$, 0·005% $CuSO_4.5H_2O$ and 1% (v/v) 0·1 N-H_2SO_4. For quantitative work the yeast was grown on 100 ml. of medium in a 1 l. Erlenmeyer flask fitted with a ground-glass joint and connected to a gas holder filled with oxygen, through (*a*) a sterilized cotton-wool filter, (*b*) a mercury seal, (*c*) a wash bottle containing 20% (w/v) aqueous KOH, (*d*) a second sterilized cotton-wool filter connecting with the gas holder. The culture vessel contained a 6 × 1 in. test tube holding 25 ml. 60% (w/v) aqueous KOH to absorb the CO_2 produced during the growth of the yeast. As the O_2 of the culture vessel was used up fresh O_2 was automatically drawn in from the gas holder and the composition of the gas phase in the culture vessel was therefore maintained constant during growth.

In some experiments the yeast was grown anaerobically, in which cases the culture vessel, after inoculation with the yeast, was flushed out with a rapid stream of N_2 for 10 min. and then closed with a glass stopper.

The temperature of incubation was 25° and at the end of the incubation period the yeast was filtered off through a sintered-glass filter (Pyrex, porosity 3), dried at 100° and weighed.

Analytical methods

Carbon dioxide produced during growth. This was determined by titrating the KOH in the boiling tube against standard acid using phenolphthalein and methyl orange successively as indicators.

Glucose. The culture filtrate was boiled with 1% of Actibon charcoal (Hopkins & Williams) for 30 sec., allowed to stand for 10 min. and filtered. Glucose in the filtrate was estimated by the method of Shaffer & Somogyi (1933).

Volatile fatty acids were estimated either by the method of Friedemann (1938) or by distillation in the apparatus of Markham (1942) followed by titration in a CO_2-free atmosphere using phenol red as indicator.

Acetaldehyde was estimated by direct distillation into $NaHSO_3$ solution in the lactic acid apparatus of Friedemann, Cotonio & Shaffer (1927).

Glycerol was estimated using the procedure described by Platt & Glock (1943) for the estimation of inositol. A longer period of incubation was found to be necessary to ensure complete removal of the glucose (2·5–3 hr.). The results are only very approximate because of the rather uncertain correction which must be made for glycerol produced during the removal of the residual glucose.

Acetic acid, ethanol and ethyl acetate in mixtures. The estimation of these substances in mixtures containing all three was based on the methods described by Friedemann (1938) for the estimation of volatile alcohols and fatty acids. Direct application of these methods is not possible because they would involve hydrolysis of the ester. *Free acetic acid*: to 2 g. $CaCO_3$ were added 20 ml. of the culture filtrate and 100 ml. water. The mixture was gently warmed under reflux for 5 min. to complete the formation of calcium acetate and 100 ml. was then distilled into a receiver cooled in ice (distillate *A*). The residue was thoroughly cooled, 180 ml. water, 50 g. $MgSO_4.7H_2O$ and 10 ml. 10% (w/v) $HgSO_4$ in 2 N-H_2SO_4 were added followed by 2 ml. 20 N-H_2SO_4 and the mixture distilled. Distillate (200 ml.) was collected and titrated against standard alkali in a CO_2-free atmosphere using phenol red as indicator. *Ethyl acetate*: distillate *A* contained ethyl acetate and free ethanol. To *A* was added 10 ml. 20 N-H_2SO_4 and the mixture refluxed for 30 min. over an open flame. It was then thoroughly cooled, the condenser washed down with 90 ml. water, 50 g. $MgSO_4.7H_2O$ and 10 ml. 10% (w/v) $HgSO_4$ in 2 N-H_2SO_4 added and the mixture distilled. Distillate (200 ml.) was collected and the acid titrated as above. This is the acid component of the ester and is therefore equivalent in amount to the ester present. *Ethanol* was estimated as the difference between the total alcohol as determined by the method of Friedemann and the alcohol present as ester. Table 1 is representative of the results obtained in this way with known mixtures of acetic acid, ethanol and ethyl acetate. Acetaldehyde at a concentration of 1 mg./ml. did not interfere with these estimations.

Table 1. *Analysis of a known mixture of acetic acid, ethanol and ethyl acetate*

(For methods used see text.)

	In mixture (mg./ml.)	Found (mg./ml.)	Recovery (%)
Acetic acid	1·27	1·16	91
Ethanol	3·95	3·67	93
Ethyl acetate	0·90	0·80	89

Carbon built into cell substance. Determinations of total carbon in *H. anomala* grown for varying lengths of time were made using the wet ashing method of Van Slyke & Folch (1940). Values of 47·2 and 47·3% were obtained for cells

harvested after 72 and 360 hr. The carbon built into cell substance was therefore obtained by multiplying the dry weight of yeast by 0·47.

Preparation of 3:5-dinitrobenzoates from alcohols. The method is a modification of that described by Reichstein (1926).

Reagents. (1) Pure anhydrous pyridine prepared by distillation over alkaline permanganate followed by distillation over anhydrous BaO. (2) Crystallizable benzene stored over fused $CaCl_2$. (3) Ether stored over Na. (4) 3:5-Dinitrobenzoyl chloride freshly prepared from 3:5-dinitrobenzoic acid and PCl_5. Carefully heat a mixture of equal weights of the acid and PCl_5 until all $POCl_3$ has been driven off. Dissolve the residue in the dry benzene and filter through a glass filter (porosity 1): prepare just enough for immediate use.

Method. To 1 mol. of the alcohol dissolved in a little dry benzene add 1·5 mol. of 3:5-dinitrobenzoyl chloride as a 7·5% (w/v) solution in dry benzene. Mix well and add 3 mol. of anhydrous pyridine slowly and with vigorous shaking. The mixture is kept overnight at room temperature and is then diluted with 4 vol. of dry, alcohol-free ether and extracted once with 2N-HCl, the extract being discarded. It is then extracted repeatedly (at least four times) with equal volumes of 2N-NaOH until the white precipitate is dissolved, the extracts again being discarded. It is extracted once more with 2N-HCl and washed four times with water, ether being added from time to time to keep up the volume. Finally the solution is evaporated to dryness *in vacuo* and the product recrystallized from 60% (v/v) aqueous ethanol, washed with a large volume of water and dried over P_2O_5. Starting with 160 mg. of ethanol a final yield of 75% of the theoretical was obtained. The method gave no reaction with ethyl acetate that had been stored over fused $CaCl_2$.

Identification of products of metabolism of glucose

Cultures of *H. anomala* grown aerobically for 72 hr. were filtered through Whatman no. 2 paper and the clear filtrate was used for the work described below.

Acetaldehyde. The culture filtrate (3 l.) was distilled through a 50 cm. fractionating column fitted with a reflux head and the first 5 ml. of distillate were collected in 25 ml. of a saturated solution of 2:4-dinitrophenylhydrazine in 2N-HCl. This fraction had distilled below 30°. The precipitate which formed was washed successively with N-HCl, saturated aqueous Na_2CO_3, water, N-HCl and finally water. It was recrystallized from 80% (v/v) aqueous ethanol (orange plates) and dried over P_2O_5 *in vacuo* for several days. M.p. 157–160° (uncorr.). The mixed m.p. with a sample of acetaldehyde 2:4-dinitrophenylhydrazone (m.p. 155–158°) prepared from freshly distilled acetaldehyde was 155–157°. (Found: C, 43·1; H, 3·7; N, 24·7. Calc. for $C_8H_8N_4O_4$: C, 42·8; H, 3·6; N, 25·0%.)

Acetic acid. The culture filtrate (12 l.) was adjusted to pH 8·0 with 15N-NaOH and evaporated *in vacuo* to 400 ml. Water (500 ml.) was added followed by 10N-HCl until the solution was red to thymol blue (pH 1–2). The solution was distilled to dryness *in vacuo*, the distillate being collected in NaOH solution. The distillate was brought to pH 8·0 with H_2SO_4 and evaporated to dryness on a boiling-water bath. The dry residue was moistened with water, excess anhydrous $KHSO_4$ added and the mass extracted with ether four times. The ether extracts were pooled and titrated with N-NaOH using phenol red as indicator. The neutral solution was diluted to 100 ml. and examined for volatile fatty acids on silica gel columns as described by Elsden (1946) and by Moyle, Baldwin & Scarisbrick (1948). Only one band was obtained, in the position corresponding to acetic acid. The addition of acetic acid to the unknown acid again resulted in only one band in the acetic acid position, whereas the addition of propionic acid gave rise to a second, faster moving band No evidence was obtained, therefore, for the presence of any volatile fatty acid other than acetic acid in the culture filtrate.

A portion of the neutralized volatile acid solution was acidified, distilled and the distillate shaken with excess $AgCO_3$. The mixture was filtered, evaporated to small bulk and left at 0° to crystallize. The product was recrystallized twice from water and dried over P_2O_5 *in vacuo*. It crystallized as shining white plates. (Found: C, 14·9; H, 1·9; Ag, 64·2. Calc. for $C_2H_3O_2Ag$: C, 14·4; H, 1·8; Ag, 64·7%.)

Ethyl acetate and ethanol. It is extremely difficult to separate ethyl acetate and ethanol from each other in aqueous solution on a small scale because of the formation of azeotropic mixtures. Therefore identification of ethyl acetate has been done by indirect methods. It has been shown that the acid component of the ester-alcohol mixture is exclusively acetic acid and that the alcohol component is exclusively ethanol. It therefore follows that the ester must be ethyl acetate.

Culture filtrate (3 l.) was adjusted to pH 8·0 with aqueous NaOH and 300 ml. were distilled through a fractionating column as described previously, the first 5 ml. being discarded. The neutral distillate was refractionated twice, in the final distillation the fraction distilling below 81° being collected (distillate *B*). A portion of *B* was dried over anhydrous BaO and redistilled. This material (0·2 ml.) was esterified with 3:5-dinitrobenzoyl chloride as described above. The product had m.p. 93–93·7° (uncorr.). The mixed m.p. with an authentic sample of ethyl 3:5-dinitrobenzoate (m.p. 93–94°) was 93–93·5°.

Analysis of *B* showed it to consist of 30% free ethanol, 60% ester and 10% water. A portion of *B* was hydrolysed by heating at 100° for 30 min. in 0·25N-NaOH and half of the mixture was distilled off (distillate *C*). The residue was neutralized and distilled from $HgSO_4$ and H_2SO_4 as described by Friedemann for the estimation of volatile acids. The distillate was neutralized and examined for fatty acids on the silica gel chromatogram. Only one band was observed, corresponding to acetic acid.

C, which contains the alcohols liberated from ester combination as well as those originally uncombined, was oxidized with $K_2Cr_2O_7$ as described by Friedemann. The volatile acids produced were recovered by distillation and examined on the silica gel chromatogram. Again only one band was observed, corresponding to acetic acid.

These results indicate that the ester produced when *H. anomala* grows on glucose is very largely ethyl acetate. The presence of other esters in traces cannot be excluded, although none was detected.

A small amount of unidentified non-volatile, ether-soluble acid was formed in an amount equivalent to about 2 ml. of 0·1N-NaOH/mol. glucose metabolized. It gave no reaction with succinic dehydrogenase and no tests for pyruvic or lactic acids, but was not further investigated. Neither pyruvic acid nor lactic acid could be detected in the culture filtrates by a variety of colour tests nor, in the case of pyruvic acid, by the use of carboxylase.

Table 2. *Products of the aerobic and anaerobic growth of* Hansenula *species on a yeast extract-salts-4 % (w/v) glucose medium at 25°*

	mmol. product/100 mmol. glucose utilized				
	H. anomala		*H. saturnus*		
			Aerobic		
	Aerobic 3 days	Anaerobic 4 days	2 days	3 days	Anaerobic 6 days
Residual glucose	22·0	2·8	14·3	3·3	29·0
CO_2	237·0	176·0	291·0	338·0	220·0
Ethyl acetate	25·1	1·1	3·6	4·2	1·4
Ethanol	19·0	87·0	13·1	2·3	124·0
Acetic acid	3·2	2·9	1·2	0·4	1·0
Acetaldehyde	0·05	0·23	0·1	0·08	0·36
Glycerol	2·7	5·6	7·0	8·5	10·6
Cell carbon (mg. atoms)	95·0	32·0	137·0	209·0	41·0
Percentage glucose carbon accounted for	81	67	84	92	92

Quantitative analysis of the cultures

The products of the aerobic and anaerobic growth of *H. anomala* and *H. saturnus* on a glucose-yeast extract-salts medium are shown in Table 2.

The dependence of ethyl acetate formation on aerobic growth conditions is clearly shown in the case of *H. anomala*. *H. saturnus* appears to possess a much more active oxidation system than *H. anomala*, as is shown by the smaller amounts of ester and ethanol and the larger amounts of CO_2 and cell carbon that are formed aerobically. Anaerobically the main product with both yeasts is ethanol and there is a much smaller proportion of glucose carbon built into cell substance.

SUMMARY

1. Ethyl acetate, ethanol, acetic acid and acetaldehyde have been identified as products of the metabolism of glucose by *Hansenula anomala*.

2. *H. anomala* produces about 25 mmol. ethyl acetate/100 mmol. glucose utilized under aerobic growth conditions.

3. *H. saturnus* produces more carbon dioxide and cell substance and less ethyl acetate and ethanol than does *H. anomala* under similar growth conditions.

4. The formation of ethyl acetate is an aerobic process.

We wish to express our thanks to the Medical Research Council for full-time training grants to two of us (J. L. P. and J. F. W.), to Gonville and Caius College for a studentship (J. F. W.) and to Trinity College, Cambridge, for an exhibition (J. L. P.).

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Ester Formation by Yeasts

2. FORMATION OF ETHYL ACETATE BY WASHED SUSPENSIONS OF *HANSENULA ANOMALA*

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Ester formation by growing cultures of certain yeasts has been reported by Davies, Falkiner, Wilkinson & Peel (1951) in the preceding paper. The amounts of ethyl acetate, ethanol and acetic acid present in a typical culture are given in Table 1.

Table 1. *Analysis of a culture of* Hansenula anomala

(Culture of vol. 100 ml. grown aerobically for 72 hr. at 25° on a medium containing 4% (w/v) glucose, salts and Difco yeast extract.)

Substance	Concentration ($M \times 10^{-3}$)
Acetic acid	5·8
Ethanol	35
Ethyl acetate	46

It is possible to calculate the concentration of ester which would be in equilibrium with the observed concentrations of ethanol and acetic acid, if the ester were formed by the condensation:

$$CH_3COOH + C_2H_5OH = CH_3COOC_2H_5 + H_2O.$$

Using the equilibrium data quoted by Parks & Huffman (1932), a value of 0·000015 M is obtained.

The concentration of ester actually found was 0·046 M. It may be concluded that the ester is not formed by the condensation indicated above but by some different process. The present communication reports investigation of the process involved in the formation of ester by washed suspensions of cells.

METHODS

Organism, growth media and preparation of washed suspensions. The organism used throughout was *Hansenula anomala* Sydow (no. 119) obtained from the Carlsberg Laboratory. The yeast was grown on the medium described by Davies *et al.* (1951). The sterilized medium was inoculated from a culture grown for 24 hr. at 25° on a similar medium but with 5% glucose. Except where otherwise specified, all cultures were grown for 48 hr. at 25°. Two growth methods were employed: when small quantities of yeast were required, cultures were grown in Roux bottles and the pellicle which formed was removed by filtration through several layers of muslin. Alternatively the yeast was grown in a stirred, aerated vessel as described by Mitchell (1949). Usually 2 l. of medium were contained in a 5 l. flask. This method produces a pellicle-free culture and was used when larger quantities of yeast were required. No significant differences in ester formation were observed between cultures grown by the two methods.

The yeast was separated from the pellicle-free culture by centrifugation, washed once with water and resuspended in water. The suspensions settled quite rapidly, consequently thorough mixing was necessary before removing samples. Dry weight determinations were made turbidimetrically, after the yeast had been dispersed by the addition of 10% (v/v) of 0·05 M-phthalate buffer pH 3 containing 0·1% Teepol X (Imperial Chemical Industries).

Ester determinations. The method used was developed from that described by Keenan (1945) for the determination of ethyl acetate in methanolic solution, which is based on the conversion of the ester to the corresponding hydroxamic acid by the action of alkaline hydroxylamine:

$$CH_3COOC_2H_5 + NH_2OH = CH_3CONHOH + C_2H_5OH.$$

The amount of hydroxamic acid formed is measured by the colour of its ferric complex in a manner similar to that used by Lipmann & Tuttle (1945) in the determination of acetyl phosphate. Consistent results could not be obtained when the method as described by Keenan was applied to aqueous solutions of ethyl acetate. A detailed investigation of the conditions affecting the colour intensity of the ferric complex and also of those affecting hydroxamic acid formation was therefore carried out.

Unknown to the author a similar investigation had been carried out by Hestrin (1949) in connexion with the determination of acetylcholine. As the author's results are in almost complete agreement with those of Hestrin no account will be published.

The procedure adopted was as follows: 2·0 ml. of 2·5 N-NaOH were added to 2·0 ml. of 2·0 M-NH_2OH.HCl, followed by 4·0 ml. of the sample containing up to 10 μmol. of ester. After standing for 5–10 min., 1·0 ml. of 5·6 N-HCl was added, followed by 1·0 ml. of 15% (w/v) $FeCl_3.6H_2O$ in 0·2 N-HCl, mixing after each addition. The colour intensity was measured using the Hilger 'Spekker' absorptiometer with Ilford no. 604 filters. A linear relationship between extinction coefficient and the amount of ester present was obtained up to 10 μmol. of ester. A separate calibration curve was constructed for each new batch of reagents, using a freshly prepared aqueous solution of ethyl acetate as standard. Determinations on aqueous samples of ester containing 6–10 μmol. were reproducible to within 3%.

As far as is known the only compounds giving the colour reaction under these conditions are esters and acid anhydrides. When high concentrations of acetaldehyde

(e.g. 0·1 M) were present in the test sample, a slow increase in colour intensity was observed, extending over several minutes after addition of the HCl and $FeCl_3$ reagents. This was presumably due to a slow formation of hydroxamic acid from the acetaldehyde and was corrected for by taking successive colorimeter readings at known times after addition of the HCl and $FeCl_3$. The value at zero time was then deduced by extrapolation.

The absence of acyl phosphate in samples can be checked by reversing the order of addition of the NaOH and the HCl, as suggested by Hestrin (1949). No acyl phosphate has been detected in suspensions of *H. anomala* during ester formation.

Separation of the ester from reaction mixtures. Initially the separation was effected by carrying out a small-scale distillation in presence of excess $CaCO_3$. The sample, containing up to 20 μmol. of ester, was diluted to 15 ml., excess powdered $CaCO_3$ added and the first 10 ml. distillate collected in a receiver cooled in ice water. To minimize frothing, 2 drops of medicinal liquid paraffin were added to the mixture and distillation carried out in a wide boiling tube heated by a microburner. About 5 mg. of talc were also added to prevent bumping. A suitable sample of the distillate was taken for ester determination. The recovery of the ester in the distillate was not complete, but was constant at 93±7%. When this method of separation is used the determination is, as far as is known, specific for volatile esters.

It was subsequently found sufficient in most cases to remove the yeast from reaction mixtures by filtration and carry out determinations directly on the filtrate. The only substance in the filtrate known to interfere with hydroxamic acid determinations was phosphate (Lipmann & Tuttle, 1945). However, direct tests showed that, under the conditions used, the amount of phosphate present did not interfere with the determinations to any significant extent.

Total alcohol determinations. The method used was a modification of that due to Northrop, Ashe & Senior (1919), in which the alcohol is oxidized by $K_2Cr_2O_7$ and the residual $K_2Cr_2O_7$ determined iodometrically. A preliminary distillation in the presence of alkaline HgO was carried out to remove acids, aldehydes and ketones.

To the sample, containing up to 100 μmol. of alcohol, were added 1·0 ml. of 5 N-NaOH, 1·0 ml. of 10% (w/v) $HgSO_4$ in 2 N-H_2SO_4 and water to a total volume of 15 ml. The mixture was allowed to stand at room temperature in a stoppered flask for 15–20 min. to hydrolyse any ester present. It was then heated slowly to boiling point and 10 ml. distillate were collected. The distillate was added to a cooled mixture of 4·0 ml. of 1% (w/v) aqueous $K_2Cr_2O_7$ and 1·0 ml. of H_2SO_4 contained in a boiling tube. The tube was closed with a loose-fitting stopper or glass bulb, immersed in a boiling-water bath for exactly 20 min., then cooled in running water for about 5 min., 4·0 ml. of 10% (w/v) aqueous KI added and the liberated I_2 titrated against 0·03 N-$Na_2S_2O_3$. The method was calibrated empirically using a standard ethanol solution. A linear relationship between the $Na_2S_2O_3$ titre and the amount of ethanol present was obtained over the range 0–100 μmol. ethanol. Over this range there was found a decrement in titre of 0·00376 m-equiv. $Na_2S_2O_3$/μmol. of ethanol present. Duplicate determinations on 60–100 μmol. of ethanol agreed to within 3%.

Measurement of ester synthesis. Except where otherwise indicated, ester synthesis was measured as follows:

Total liquid volumes of 10·0 ml. containing buffer, washed yeast and substrate were incubated in 100 ml. Erlenmeyer flasks of uniform shape. The flasks were closed with rubber bungs to prevent loss of ester and were shaken in identical fashion throughout the incubation period. Variation of the rate of shaking produced a marked effect on the yield of ester. After incubation for 1 hr. the yeast was removed by filtration and determinations carried out as described above.

Under these conditions the amount of ester initially present could be neglected and the amount of ester found after incubation taken as the ester formed.

Acetate, where present in incubation mixtures, was added as a mixture of acetic acid and sodium acetate in such proportions as to give the required pH. In experiments at pH 2·8 where McIlvaine buffer was added, the standard citrate-phosphate solution at pH 2·5 was added, this giving a pH of 2·8 in the final volume.

RESULTS

The earlier experiments were carried out using ethanol in presence of 0·04 M-acetate as substrate. It was subsequently found that higher concentrations of acetate inhibit ester formation and several experiments were repeated using ethanol alone as substrate. The results were substantially the same in both series of experiments except that the pH optimum for ester formation is altered by the presence of acetate. Table 2 illustrates the ability of a washed suspension of *H. anomala* to form ester from ethanol in the presence of acetate. In the absence of ethanol no ester was formed. In the absence of acetate, ester formation was diminished, but this may have been due to the change in pH optimum found when ethanol alone replaced (ethanol + acetate) as substrate.

Table 2. *Formation of ester by a washed suspension of* Hansenula anomala

(The complete system comprised, in a total vol. of 5·0 ml.: McIlvaine buffer (pH 4·6), 1·5 ml.; acetate, 0·04 M; ethanol, 0·1 M; washed yeast, 80 mg. dry wt./ml. Incubated with shaking for 1 hr. at 25°.)

System	Ester formed (μmol.)
Complete system	230
Control (boiled yeast)	20
Ethanol absent	18
Acetate absent	128
Ethanol and acetate absent	14

Stability of activity. The ester-forming activity of washed suspensions was unstable. On keeping for 24 hr. at 1°, 50–75% of the initial activity was lost.

Effect of age of culture. The activity of washed suspensions in producing ester from (ethanol + acetate) increased with the period of growth of the cultures prior to harvesting, up to 48 hr. Cells harvested after 96 hr. were much less active (Table 3).

Dependence of ester formation on the presence of oxygen. Table 4 shows that ester formation from (ethanol + acetate) was dependent upon the presence of oxygen.

Table 3. *Effect of age of culture on ester formation by a washed suspension of* Hansenula anomala

(Yeast harvested after growth at 25° for the times indicated. Samples of washed cells of about 90 mg. dry weight together with McIlvaine buffer (pH 4·6), 3·0 ml., 0·04 M-acetate and 0·15 M-ethanol in a total vol. of 10·0 ml., incubated with shaking for 1 hr. at 25°.)

Age of culture (hr.)	Ester formed (μmol./100 mg. dry weight yeast)
12	0
24	6
48	98
96	25
336	7

Table 4. *Dependence of ester formation by a washed suspension of* Hansenula anomala *on the presence of oxygen*

(Krebs vessels contained, in a total vol. of 10·0 ml.: McIlvaine buffer (pH 4·6), 3·0 ml.; acetate, 0·04 M; ethanol, 0·1 M; washed yeast, 4·0 mg. dry wt./ml. Incubated with shaking for 1 hr. at 25°.)

Gas phase	Ester formed (μmol.)
N_2	5
Air	33
O_2	43

Effect of pH. The precise relationship between pH and ester formation is a little uncertain. With (ethanol + 0·04 M-acetate) as substrate and in presence of McIlvaine buffer, ester formation proceeded at a maximum rate at pH 4·5–5·0 (Fig. 1). The situation was complicated, however, by the fact that acetate was inhibitory at concentrations above 0·05 M.

With ethanol alone as substrate the optimum pH was found to vary with the nature of the buffer present. The results for glycine, phthalate, phosphoric acid-potassium dihydrogen phosphate, and McIlvaine citrate-phosphate buffers are shown in Fig. 1. Ester formation from ethanol alone was studied at pH 2·8 in presence of McIlvaine buffer.

Effect of concentration of yeast cells. Ester formation was found to be directly proportional to the concentration of yeast cells over the range 0–9 mg. dry wt./ml. both in presence of ethanol alone (at pH 2·8) and of (ethanol + 0·04 M-acetate) (at pH 4·6).

Effect of ethanol concentration. Fig. 2 shows the effect of ethanol concentration on ester formation in presence and in absence of 0·04 M-acetate at pH 4·6, and in the absence of acetate at pH 2·8. In presence of 0·04 M-acetate at pH 2·8 ester formation was completely inhibited (Fig. 3). In each of the three cases studied, increasing the ethanol concentration above 0·2 M produced little increase in ester formation.

Effect of acetate concentration. At pH 4·6, the addition of 0·008 M-acetate produced 50 % increase in the production of ester, but the rate decreased with further increase in acetate concentration until complete inhibition was obtained in presence of about 0·1 M-acetate. At pH 2·8, a small stimulation (7 %) was observed on addition of 0·01 M-acetate. Further increase in acetate concentration produced inhibition which was complete at a lower concentration (0·04 M) than at pH 4·6 (Fig. 3).

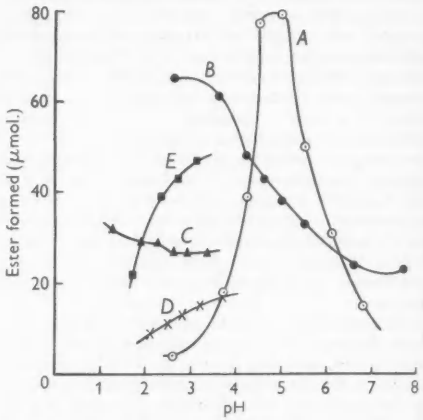

Fig. 1. Effect of pH on ester formation by washed suspensions of *Hansenula anomala*. Incubation mixtures contained in a total vol. of 10·0 ml.: ethanol, 0·2 M; washed yeast, 40 mg. dry wt. (Exps. *A*, *C*, *D*, *E*) or 25 mg. dry wt. (Exp. *B*); buffers as follows: Exp. *A*, 0·04 M-acetic acid-sodium acetate mixtures together with 3·0 ml. McIlvaine standard citrate-phosphate mixtures; Exp. *B*, 3·0 ml. McIlvaine standard citrate-phosphate mixtures; Exp. *C*, 3·0 ml. Sørensen standard glycine in NaCl-HCl mixtures (Clark, 1928); Exp. *D*, 0·25 M-potassium phthalate buffer; Exp. *E*, 0·03 M-H_3PO_4-KH_2PO_4 mixtures. Incubated with shaking for 1 hr. at 25°. pH of incubation mixtures determined using glass electrode. Since the activities of the yeast suspensions used varied somewhat in different experiments, only the shapes and not the heights of the different curves are comparable.

Effect of temperature. Ester formation occurred readily over the range 19–30° with an optimum at 25–30°. There was a sharp drop in activity at 37° (Table 5).

Ester formation from other substrates. When incubated for 1 hr. under conditions optimal for ester formation from ethanol alone, ester formation could be shown from equimolar concentrations of glucose, but not from methanol, *n*-propanol, *iso*-propanol, *sec.*-butanol, choline, glycol, or glycerol. Traces, of doubtful significance, were found in presence of *n*-butanol, *iso*butanol, *n*-amyl alcohol, and benzyl alcohol. When added to the normal

ethanol substrate at pH 2·8 or 4·6, 0·01 M-acetaldehyde stimulated, whereas 0·2 M-acetaldehyde inhibited ester production. No appreciable ester was formed from acetaldehyde alone, aerobically or anaerobically at either pH.

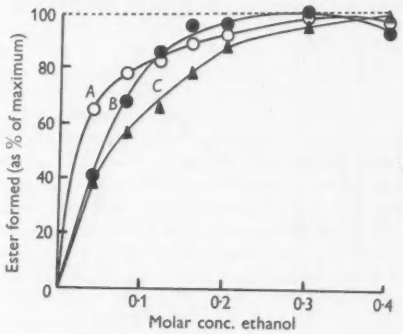

Fig. 2. Effect of ethanol concentration on ester formation by a washed suspension of *Hansenula anomala*. Incubation mixtures of total vol. 10·0 ml. contained: McIlvaine buffer (pH 2·5 or 4·6), 3·0 ml.; washed yeast, 60 mg. dry wt. (Exp. *A*, *C*) or 25 mg. dry wt. (Exp. *B*); acetate, 0·04 M (Exp. *C* only); ethanol as indicated. Incubated with shaking for 1 hr. at 25°. Exp. *A*: acetate absent, pH 2·8; Exp. *B*: acetate absent, pH 4·6; Exp. *C*: acetate present, pH 4·6.

Course of ester formation from ethanol alone. Fig. 4 shows the progress curve for ester formation from ethanol alone at an initial concentration of 0·2 M: the production is approximately linear for the first 2 hr.

Table 5. *Effect of temperature on ester formation by a washed suspension of* Hansenula anomala

(Duplicate mixtures were incubated with shaking for 1 hr. at the various temperatures. Incubation mixtures of total vol. 10·0 ml. contained: McIlvaine buffer, 3·0 ml.; ethanol, 0·2 M; acetate, 0·04 M (Exp. 1 only); washed yeast, 4·0 mg. dry weight/ml. (Exp. 1) or 4·5 mg. dry weight/ml. (Exp. 2).)

	Ester formed (μmol./100 mg. dry wt. yeast)	
	Exp. 1	Exp. 2
Temperature (°)	(pH 4·6; substrate: ethanol + acetate)	(pH 2·8; substrate: ethanol)
19	58	47
25	110	60
30	78	69
37	10	16

When smaller initial concentrations of ethanol were used in the presence of an adequate oxygen supply, ester was found to accumulate until the free alcohol had almost disappeared. Beyond this point the ester disappeared very rapidly (Fig. 5). This behaviour was later used to obtain a sample of the ester relatively free from ethanol.

Identification of the ester formed. The identification of the ester is based on indirect evidence and depends upon the separation and identification of the acid and alcohol moieties.

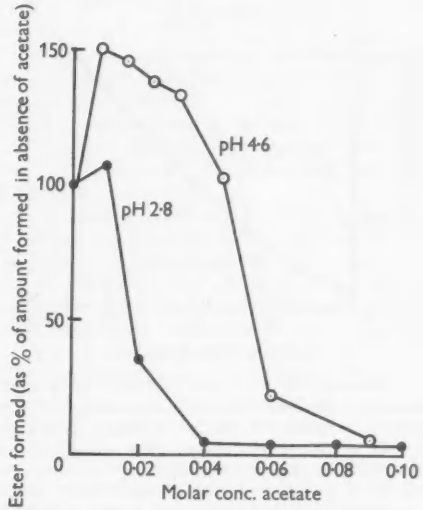

Fig. 3. Effect of acetate concentration on ester formation by a washed suspension of *Hansenula anomala*. Incubation mixtures of total vol. 10·0 ml. contained: McIlvaine buffer (pH 2·5 or 4·6), 3·0 ml.; washed yeast, 45 mg. dry wt. (Exp. at pH 2·8) or 80 mg. dry wt. (Exp. at pH 4·6); ethanol, 0·2 M; acetate as indicated. Incubated with shaking for 1 hr. at 25°.

The ester could not be readily separated from the unchanged free ethanol in the reaction mixture. Consequently it was desirable to stop the reaction at a point where the free ethanol concentration was as low as possible relative to the ester concentration. Under the conditions used the amount of ester present decreases rapidly when the free alcohol in the mixture has almost disappeared. In these circumstances, the relatively slow alcohol determinations were of little use for determining the optimum incubation time. The procedure adopted, therefore, was to follow the course of the reaction by ester determinations until the amount of ester present began to fall. At this point the reaction was stopped and the ester separated.

The reaction mixture contained in 200 ml. water: 1·32 g. dry weight of washed yeast; 60 ml. of McIlvaine buffer (pH 2·5); approximately 7 mmol. of ethanol. This mixture was contained in a 5 l. flask closed with a rubber bung and rotated in the apparatus described by Mitchell (1949). Samples were removed periodically and the ester present immediately determined. Total alcohol was also determined on some of the samples.

When the amount of ester present began to fall, excess powdered $CaCO_3$ and about 5 mg. of talc were added, and all but 30–40 ml. distilled, the receiver being cooled in ice water. Samples of the distillate were removed for alcoho land ester

determinations and then 10 ml. of 5N-NaOH added to the remainder which was left overnight to complete the hydrolysis of the ester. 10 ml. of 10% (w/v) $HgSO_4$ in 2N-H_2SO_4 and 20–30 ml. water were then added and the whole distilled, 100 ml. distillate being collected. This distillate (*A*), now free from aldehydes, ketones and formic acid, contained the alcohol moiety of the ester together with any residual ethanol.

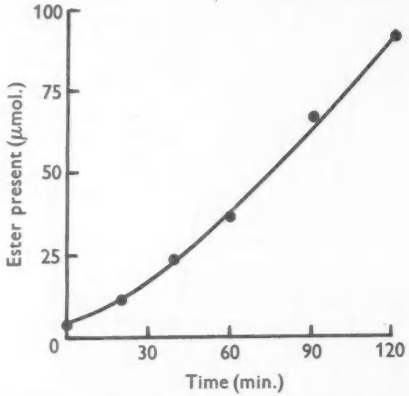

Fig. 4. Progress curve of ester formation by a washed suspension of *Hansenula anomala* in presence of excess ethanol. Duplicate incubation mixtures of total vol. 10·0 ml. contained: McIlvaine buffer (pH 2·5), 3·0 ml.; ethanol, 0·2M; washed yeast, 60 mg. dry weight. Incubated with shaking at 25°, one of the duplicate mixtures being used for ester determination at each of the times indicated.

To the residue, containing the Na salt of the volatile acid moiety of the ester, were added in turn, 100 ml. of water, about 5 mg. of talc, 50 g. of $MgSO_4.7H_2O$, 10 ml. of 20N-H_2SO_4, and a further 80 ml. of water with careful mixing. The whole was heated slowly to boiling and distilled to the point of crystallization. The distillate was diluted to 250 ml. and the acid present determined by titration of a 25 ml. sample. The remainder was neutralized, concentrated and examined chromatographically as described by Elsden (1946).

To the distillate *A* were added 2 g. of powdered $K_2Cr_2O_7$ and 10 ml. of 20N-H_2SO_4, and the mixture was heated for 30 min. under reflux in a boiling-water bath. After cooling, the condenser was washed down with 50 ml. of water, talc was added, followed by 50 g. of $MgSO_4.7H_2O$ and the whole distilled to the point of crystallization. The distillate was diluted to 250 ml. and the acid present determined by titration of a 25 ml. sample. The remainder was neutralized, concentrated and examined chromatographically. Table 6 shows the recovery of acids from the original distillate.

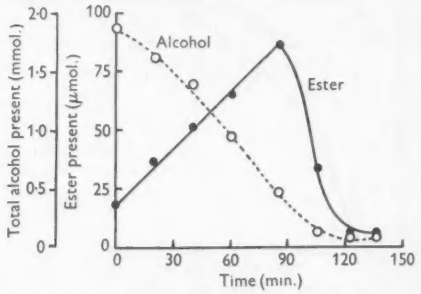

Fig. 5. Formation and disappearance of ester when a washed suspension of *Hansenula anomala* acts upon a small amount of ethanol under highly aerobic conditions. Incubation mixture of total vol. 60 ml. contained: McIlvaine buffer (pH 2·5), 18 ml.; ethanol, about 0·03M; washed yeast, 312 mg. dry weight. The mixture was incubated in a closed 5 l. flask, and rotated in the apparatus described by Mitchell (1949). Samples were removed periodically for determinations of ester and total alcohol.

Chromatographic examination showed that the acid moiety of the ester and the acid derived by oxidation of the free and ester-bound alcohol each gave rise to a single band on the chromatogram which was only slightly mobile in chloroform containing 1% (v/v) *n*-butanol, but could be washed down the column with chloroform containing 5% (v/v) *n*-butanol. When authentic acetic acid was added to each of the two derived acids and the mixtures tested on the chromatogram the behaviour was identical with that observed with the two derived acids alone.

The chromatographic evidence, considered in conjunction with the data on the recovery of acids from the hydrolysis of the ester and oxidation of the alcohols (Table 6), indicates that only one ester is formed and that this ester is ethyl acetate.

Table 6. *Recovery of volatile acids derived from the residual alcohol and the ester formed from ethanol by a washed suspension of* Hansenula anomala

(The incubated mixture was distilled and determinations of ester and total alcohol were carried out on the distillate. The volatile acids subsequently derived from the acid moiety of the ester, and from the residual alcohol together with the alcohol moiety of the ester were determined (see Text).)

Substance	Method of determination	Amount found (μmol.)
Ester after incubation	Conversion to hydroxamic acid	533
Ester-bound acid recovered	Titration with NaOH	530
Total alcohol after incubation	Oxidation by $K_2Cr_2O_7$	640
Acid recovered after oxidation of free and ester-bound alcohol	Titration with NaOH	625

DISCUSSION

The results obtained show that, as with growing cultures of the yeast, the amounts of ester formed by washed suspensions are too large to be accounted for by the reversal of a simple esterase reaction, indicating a more exergonic mechanism of ester formation. This is confirmed by the fact that, under anaerobic conditions, no ester is formed from ethanol plus acetate or from acetaldehyde, showing that oxidation is necessary for the synthesis of ester.

In the case of ester formation from (ethanol + acetate), there is no evidence that the acid moiety of the ester is derived from the added acetate. The marked stimulation of ester production followed by inhibition, occurring when the acetate concentration is increased at pH 4·6, may be due to an alteration of the external pH required to give optimal internal conditions for ester formation from ethanol alone. This explanation would also account for the effect of increasing the acetate concentration at pH 2·8 and the behaviour in presence of different buffers. The problem of the origin of the acetate moiety of the ester could be readily investigated by suitable isotope experiments.

It is possible that oxidation of ethanol may lead to the formation of a labile acetyl derivative (cf. Stadtman & Barker, 1949; Black, 1950) which then reacts with further ethanol to produce the ester. This hypothesis can only be tested by investigation of ester synthesis in a cell-free system. Attempts to prepare such a system are being made.

SUMMARY

1. Under aerobic conditions, washed suspensions of *Hansenula anomala* form ester from ethanol, alone or in presence of acetate.

2. No ester is formed in the absence of oxygen.

3. The ester formed is ethyl acetate.

4. The ester formed disappears after exhaustion of the ethanol substrate.

5. The effects of pH, age of culture, ethanol concentration and acetate concentration on ester formation have been investigated.

6. A number of alcohols have been tested but ester is formed at an appreciable rate only from ethanol or glucose. Acetaldehyde does not give rise to any ester, but in low concentrations acetaldehyde stimulates ester formation in presence of ethanol.

I wish to express thanks to my colleagues, in particular to Dr R. Davies and Dr E. F. Gale, for advice, criticism and encouragement during the course of this work. My thanks are also due to the Medical Research Council for a training grant, and to Trinity College, Cambridge, for an exhibition.

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Comparative Studies of 'Bile Salts'

2. PYTHOCHOLIC ACID

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It was shown in Part 1 (Haslewood & Wootton, 1950*b*) that the bile of three species of Boidae contained a new acid, $C_{24}H_{40}O_5$, then called by us pythocholic acid, which was easily converted into pythocholic lactone, $C_{24}H_{38}O_4$. In the African python at least, this acid, conjugated with taurine, formed the main constituent of the bile salts.

The work now described had two aims; to explore further how far pythocholic acid is characteristic of the bile of the Boidae, and to elucidate as far as possible the chemistry of the new acid.

A preliminary communication on this work has appeared (Haslewood & Wootton, 1950*a*).

RESULTS

Biological. Approximate figures for the content of pythocholic acid in the total (hydrolysed) 'bile acids' from the bile of seven species of Boidae are

Table 1. *Bile acids of Boidae*

(Approximate amounts of cholic and pythocholic acids in the 'bile acids' (see text, p. 69) of seven species of Boidae. Based on the isolation of crystalline material (methyl pythocholate, pythocholic lactone, cholic acid). Classification of snakes after Smith, 1927.)

Species	Pythocholic acid (percentage of 'bile acids'; approx. minimal figures)	Cholic acid (Hammarsten test, or approx. percentage of 'bile acids')
Green tree boa, *Boa canina*	28	3
Common boa, *Constrictor constrictor*	51	H-test, negative
Emperor boa, *C. imperator*	54	H-test, negative
Western boa, *C. occidentalis*	52	4
Rainbow boa, *Epicrates cenchris*	53	H-test, ?negative
Indian python, *Python molurus*	70	H-test, negative
African python, *P. sebae*	70	H-test, negative

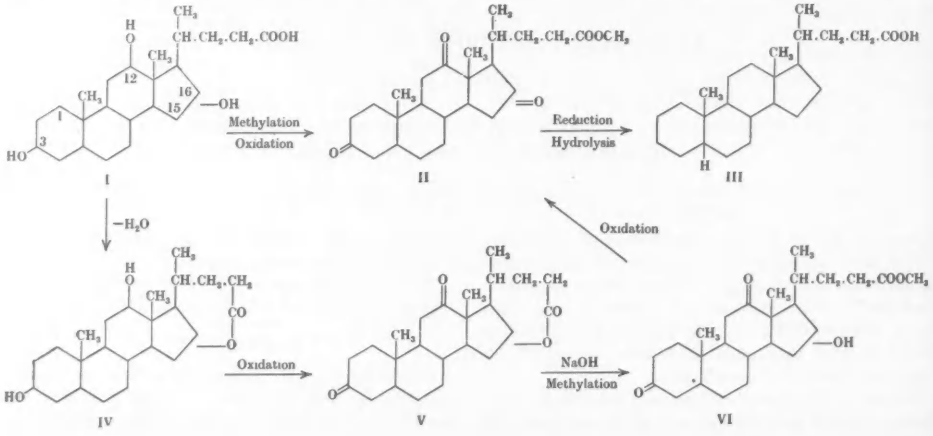
given in Table 1, together with an indication of the presence or absence of cholic acid in these biles. Cholic acid was isolated from the bile of two species: a positive Hammarsten test thus probably indicates its presence in the bile of other Boidae.

Chemical. Results of the experiments described in full below can be interpreted as follows.

Pythocholic acid $C_{24}H_{40}O_5$ (I) formed a methyl ester which was oxidized by chromic oxide to the triketone methyl dehydropythocholate (II); and this was reduced by the Kishner-Wolff method to cholanic acid (III). Hence pythocholic acid contains three secondary hydroxyl groups attached to the cholanic acid nucleus.

Pythocholic lactone (IV) formed a diketone, dehydropythocholic lactone (V) on chromic oxidation. It also gave a diacetyl derivative, which could be recovered unchanged after treatment with chromic oxide, and a monoacetate, the free OH group of which was oxidized to give a monoketone, characterized by a semicarbazone. This behaviour, together with the fact that pythocholic acid does not respond to the Hammarsten test, strongly suggests that one of the two hydroxyl groups in pythocholic lactone is at C_{12}; on biogenetic grounds the other may be at C_3. This suggestion is not contradicted by the optical findings, for dehydrogenation of hydroxyl groups at C_3 and C_{12} in deoxycholic acid produced an increase in molecular rotation of roughly the same amount as was found when pythocholic lactone was converted to the dehydro-compound. $[M]_D$3:12-diketocholanic acid (dehydrodeoxycholic acid) $-$ $[M]_D$3α:12α-dihydroxycholanic acid (deoxycholic acid) $= 349° - 153° = +196°$. $[M]_D$ dehydropythocholic lactone $-[M]_D$ pythocholic lactone $= 448° - 203° = +245°$. 3:11-Dihydroxy compounds show a small rotation change on oxidation to diketones, while 3:6- and 3:7-dihydroxy compounds show considerable negative rotation changes (for summary of data, see Barton & Klyne, 1948).

In the conditions used, opening of the lactone ring in dehydropythocholic lactone (V), followed by methylation, led to molecular changes; in one experiment it was possible to isolate, in small yield, an impure compound, probably (VI), which was converted to methyl dehydropythocholate (II) and thence to cholanic acid (III). In another case, however, the crystalline intermediate compound could not be converted to methyl dehydropythocholate; it was apparently an isomer of (VI).



Partial oxidation of methyl pythocholate followed by Kishner-Wolff reduction of the product gave a mixture from which was isolated an acid, apparently one of the expected deoxypythocholic acids, $C_{24}H_{40}O_4$, formed by elimination of a hydroxyl group at C_3 or C_{12} from pythocholic acid.

On the assumption that two OH groups in pythocholic acid are at C_3 and C_{12}, and by comparison with methyl deoxycholate ($[M]_D + 195°$; Charonnat & Gauthier, 1947), it can be calculated that the molecular rotation difference due to inclusion of the lactone-forming OH group in methyl pythocholate is approximately $-72°$. Dehydrogenation of this group produced the $[M]_D$ difference of $-341°$, derived as follows:

	$[M]_D$
Dehydrogenation of two OH groups not concerned in lactonization (see below)	$+245°$
Methyl dehydropythocholate	$-96°$
Change due to dehydrogenation of lactone-forming OH group	$-341°$

EXPERIMENTAL

General. Melting points are uncorrected. Optical rotations were determined in a 1 dm. micro-tube. Micro-analyses (C and H) were done by Drs Weiler and Strauss, Oxford. Al_2O_3 (Hopkin and Williams) was neutralized and reactivated (Shoppee, 1949). L.p. = light petroleum, b.p. 40–60°. 20% CrO_3 = a solution made by dissolving 20 g. of CrO_3 in the minimum amount of water and making to 100 ml. with acetic acid. H-test = Hammarsten's HCl test (Haslewood, 1943).

Isolation of acids

Methyl pythocholate from bile. The bile salts (0·5–1 g.) were obtained and hydrolysed as previously described (Haslewood & Wootton, 1950*b*). The 'bile acids', precipitated with HCl and NaCl (excess) were collected at once, washed with water and dried *in vacuo* over H_2SO_4. The mother liquors in all cases were tested with $BaCl_2$ for SO_4^{--}; only traces of this ion were detected. After weighing and examination by the H-test, the dried 'bile acids' were dissolved in ethanol and this was saturated with diazomethane, carried over in N_2 from nitrosomethylurea decomposed in the usual way. The ethanolic solution was acidified with H_2SO_4, diluted with water and extracted with ether. The ether was washed with water, dil. NH_3, water, dried (Na_2SO_4) and evaporated. The residue, from the bile of all the species mentioned in Table 1, crystallized at once; it was collected, dried and weighed. After recrystallization from dilute ethanol, white needles of a hydrate were obtained. After drying at about 80° *methyl pythocholate* had m.p. 146–148°; $[\alpha]_D^{23°} = +28° \pm 1°$ in $CHCl_3$ (*c*, 1·2). $[M]_D = +123°$. (Found: C, 68·4; H, 10·4. $C_{25}H_{42}O_5$, H_2O requires C, 68·2; H, 10·0%.)

The esters obtained from six of the species of Boidae examined were identical in melting point. Mixed melting points with the esters from the African python (taken as standard) gave no depression. Yields of crude crystalline ester and/or lactone (see below) and results of the H-test are given in Table 1. Methyl pythocholate was sparingly soluble in ether and dissolved readily in $CHCl_3$ and ethanol; all crystals obtained appeared to be hydrated and from wet ether or dilute ethanol often had, before drying at about 80°, a substantially lower m.p. (approx. 115 or 135°, with effervescence) than that quoted above. Owing to the slight solubility in ether and ease of crystal formation of hydrated methyl pythocholate, the above method appeared to provide an approximately quantitative measure of the pythocholic acid in the bile salts.

In our earlier experiments, we relied on the isolation of the crystalline lactone from ethyl acetate, after preliminary warming of the crude 'bile salts' with dil. HCl. However, further work showed that the yield of lactone was very variable, depending more on the boiling of crude pythocholic acid with ethyl acetate than on allowing it to stand in acidified solutions. There seems to be no doubt that our earlier separation of ethyl esters from the bile salts of *Constrictor occidentalis* and *Python molurus* on Al_2O_3 columns led only to samples of crude ethyl pythocholate and pythocholic acid. Methyl and ethyl pythocholate appear to be easily hydrolysed on Al_2O_3 (see below).

Hydrolysis of purified methyl pythocholate (50 mg.) in ethanol (0·5 ml.) with 40% (w/v) KOH (2 drops) at 70° for 10 min. gave, after acidification, pythocholic acid which crystallized from ethyl acetate in large colourless prisms (20 mg.) having m.p. 186–187° when heated rapidly; the melting point was higher and indefinite with slow heating, presumably because of lactone formation.

Cholic acid from Constrictor occidentalis. A benzene solution of 0·306 g. of ethylated 'bile acids', prepared as described in Part 1, was poured on to a column of Al_2O_3 (3 g.). About half the material was hydrolysed on the column. 80 mg., eluted with benzene and ether/benzene, gave a negative H-test; 11 mg. eluted with ether, and 60 mg. eluted with ethanol showed a strongly positive response to this test. The latter material (60 mg.) would not crystallize, but on hydrolysis it gave, from ethyl acetate, a crystalline acid (10 mg., about 3·3% of the total 'bile acids') which, after recrystallization from ethyl acetate, formed typical prisms (5 mg., H-test, blue) of cholic acid m.p. 193–195°, not depressed by an authentic sample.

Table 2. *Separation of methyl esters from the 'bile acids' of Boa canina*

(339 mg. of esters, left from the crystallization of methyl pythocholate, on 4 g. Al_2O_3.)

Fraction	Eluent (ml. solvent)	Wt. (mg.) etc.
I	30 benzene	161 crystallized with ether H-test, negative
II	25 benzene	13·6 H-test, positive
III	50 benzene	12·3 H-test, positive
IV	25 ether	79·0 H-test, positive
V	25 ether	38·5 H-test, positive
VI	25 ether	23·3 H-test, positive

Cholic acid from Boa canina. Methyl pythocholate (0·15 g.) was crystallized from an ether solution of the methyl esters of the 'bile acids' (0·598 g.) from the bile salts (0·935 g., from one gall bladder) of this species. The mother liquors were evaporated. The residue (0·339 g.) was dissolved in benzene and poured on to Al_2O_3 (4 g.) in a wide column which was rapidly eluted as described in Table 2. Perhaps, because of rapid elution, hydrolysis was negligible. Fraction I, with ether, gave 20 mg. of pythocholic lactone. Fractions IV–VI

were combined and hydrolysed by heating in ethanol (1·5 ml.) with 40% (w/v) KOH (0·2 ml.) at about 80° for 20 min. The diluted solution was acidified with HCl and saturated with NaCl. The gummy precipitate was collected, washed and crystallized from ethyl acetate, from which cholic acid (15 mg., H-test, blue; about 2·5% of 'bile acids') was obtained. After recrystallization from ethyl acetate and drying at about 90° this had m.p. 192–194°, not depressed by authentic cholic acid.

Lactone from Constrictor constrictor. In spite of care taken to avoid conditions leading to lactonization in the isolation of methyl pythocholate from bile, this reaction could not be entirely prevented (e.g. see above, p. 69). In the case of the above species, almost the whole of the crystal crop, from ether, was composed of the lactone, m.p. 262–266°. The yield given in Table 1 is based on the weight of these crystals.

Derivatives of pythocholic acid

Methyl dehydropythocholate. This substance proved unexpectedly difficult to prepare. It was found after a number of experiments that best results were obtained when oxidation was done at about 25°. Methyl pythocholate (0·1 g.) was dissolved by warming in acetic acid (1 ml.). The solution cooled to 25°, was treated gradually with shaking, with 20% CrO_3 (0·3 ml.). After 10 min. at 25°, the solution was diluted and treated with NaCl (excess). After 24 hr. the product, which had crystallized, was collected and recrystallized from dilute ethanol from which it formed white leaflets (50 mg.), m.p. 125–132°. Elution with benzene of a benzene solution of this material from Al_2O_3 (0·5 g.) followed by recrystallization from dilute ethanol gave flat glistening laths (30 mg.) of *methyl dehydropythocholate* (II), m.p. 142–143°; $[\alpha]_D^{20°}$ – 23° ± 2° in $CHCl_3$ (*c*, 0·55). $[M]_D = -96°$. (Found: C, 72·2; H, 8·8; $C_{25}H_{36}O_5$ requires C, 72·1; H, 8·7%.)

Cholanic acid. The above substance (II) (33 mg., crystalline, partially purified) was added to a solution, in a metal bomb, of Na (40 mg.) in ethanol (2 ml.), with hydrazine hydrate (0·1 ml.). The bomb was sealed and heated at 195–205° for 4 hr. The diluted contents were then acidified with H_2SO_4 and the solid product collected, washed and crystallized three times from dilute ethanol: it (5 mg.) then had m.p. 160–161°, not depressed by authentic cholanic acid prepared by the same process from ethyl dehydrocholate. In another experiment, 20 mg. of methyl dehydropythocholate, m.p. 129–131°, gave 9 mg. of cholanic acid, m.p. 158–159°. The mixed m.p. with an authentic sample (m.p. 161–164°) was 161–163°. (Found: C, 80·1; H, 11·4. Calc. for $C_{24}H_{40}O_2$: C, 80·0; H, 11·1%.)

Dehydropythocholic lactone (V). A solution of pythocholic lactone (0·8 g., m.p. 264–266°) in acetic acid (8 ml.) was cooled in water at room temperature and treated gradually with 20% CrO_3 (1·6 ml.). After 15 min., during which the resulting precipitate was slowly dissolved with occasional shaking, the solution was diluted with about 5 vol. of water. After 24 hr. the solid was collected, washed and dried. It (0·734 g.) was crystallized from dilute ethanol and from l.p./benzene, forming long white needles of *dehydropythocholic lactone*, m.p. 239–241° (decomp.); $[\alpha]_D^{22°}$ + 116° ± 2° in $CHCl_3$ (*c*, 1·0). $[M]_D = +448°$. (Found: C, 74·6; H, 8·6. $C_{24}H_{34}O_4$ requires C, 74·6; H, 8·8%.) Pythocholic lactone had $[\alpha]_D^{22°}$ + 52° ± 1° in $CHCl_3$ (*c*, 1·2). Hence $[M]_D = +203°$.

A sample of deoxycholic acid-ether complex, prepared from cholic acid, had $[\alpha]_D^{22°}$ + 39° ± 1° in approx. 15% (v/v) ethanol/$CHCl_3$ (*c*, 1·15). Hence $[M]_D = +153°$. A highly purified specimen of dehydrodeoxycholic acid had $[\alpha]_D^{22°}$ + 90° ± 1° in $CHCl_3$ (*c*, 1·3). Hence $[M]_D = +349°$. (Deoxycholic acid had $[\alpha]_D^{22°}$ + 48° in pure ethanol.)

Diacetylpythocholic lactone. Pythocholic lactone (0·2 g.) in pyridine (1 ml.) with acetic anhydride (1 ml.) was heated at about 95° for 6 hr. The cooled mixture was diluted with aqueous HCl and the solid precipitate was collected, washed and crystallized from dilute ethanol and l.p./benzene. It was then dissolved in benzene and the solution poured on to Al_2O_3 (1 g.) in a column. The product was eluted with benzene (70 ml.) and recrystallized from l.p./benzene and benzene as long white needles. This *diacetyl pythocholic lactone* had m.p. 197–198°. (Found: C, 70·8; H, 9·0. $C_{28}H_{42}O_6$ requires C, 70·9; H, 8·9%.) This substance was recovered unchanged (m.p. 197–198°, not depressed by the starting material) after treatment with cold 20% CrO_3 in acetic acid.

Monoacetyl pythocholic lactone and derivatives. The lactone (0·12 g.) in pyridine (1 ml.) with acetic anhydride (1 ml.) was kept at about 20° for 16 hr. After dilution with aqueous HCl, the solid was collected, washed and crystallized from l.p./benzene, dilute ethanol and finally benzene; from this it gave white needles. This *monoacetyl pythocholic lactone* had m.p. 243–244°. (Found: C, 72·6; H, 9·3. $C_{26}H_{40}O_5$ requires C, 72·2; H, 9·3%.)

The above substance (27 mg., m.p. 241–243°) in acetic acid (2 ml.) was treated with 20% CrO_3 (0·1 ml.) and the mixture was allowed to stand for 5 min. The solid which separated on dilution was collected, washed and crystallized from dilute ethanol, from which it formed white leaflets. This *ketone*, had m.p. 235–237°, depressed by the original substance. (Found: C, 71·7; H, 8·6. $C_{26}H_{38}O_5$ requires C, 72·5; H, 8·8%.) In spite of the low C analysis, further crystallization did not change the melting point. The *semicarbazone*, prepared in the usual way, had m.p. 259°, with effervescence. (Found, by micro-Kjeldahl and Nesslerization: N, 8·2. $C_{27}H_{41}O_5N_3$ requires N, 8·6%.)

Opening of lactone ring. Dehydropythocholic lactone (0·4 g.) was heated at about 90° for 5 hr. with NaOH (0·1 N; 40 ml.). The compound gradually dissolved to give a yellow solution. After 24 hr. at room temperature this was acidified with H_2SO_4 and the precipitated solid collected, washed and dissolved at once in ethanol. The solution was saturated with diazomethane (in N_2) and left for 16 hr. Evaporation of the ethanol left a colourless gum which slowly crystallized. The crystals were collected and washed with l.p.; they (0·37 g.) had m.p. 123–131°. After two recrystallizations from ethanol, this compound (isomers of VI) had m.p. 156–160°; $[\alpha]_D^{21°}$ + 82° ± 2° in $CHCl_3$ (*c*, 0·83). (Found: C, 72·1; H, 9·2. $C_{25}H_{38}O_5$ requires C, 71·8; H, 9·1%.) The m.p. of the material recovered from the rotation determination was 163–165°.

In spite of the fact that the $[M]_D$ (+ 343°) of this substance agreed with what would be expected from VI, a number of attempts to convert the compound to methyl dehydropythocholate (II) entirely failed. Likewise, CrO_3 oxidation of the residue left on evaporation of the liquors from the purification of the substance yielded, even after fractionation on an Al_2O_3 column, only about 4 mg. of crystalline material of m.p. 109–111°. In another similar experiment beginning with the dehydrolactone it did prove possible, however, to isolate a very small yield of methyl dehydropythocholate (20 mg., m.p. 129–131°) which was converted to cholanic acid (see above). Other experiments suggested that it might be possible to open the lactone ring by milder alkali treatment, with ethanol as a solvent.

Partial oxidation, followed by reduction, of methyl pythocholate (cf. Haslewood, 1943). This ester (0·2 g.) was dissolved by warming with acetic acid (2 ml.) and sodium acetate ($CH_3COONa.3H_2O$; 0·4 g.). A solution of K_2CrO_4 (31·7 g./100 ml.; 0·4 ml.) was added slowly with shaking, followed by acetic acid (4 ml.). The mixture was shaken at intervals during 10 min., until all the precipitate had dissolved. It was then left with occasional shaking at about 23° for 24 hr., after which it was diluted with water and treated with NaCl (excess). After 24 hr. the organic product was extracted with ether and this was washed with water, dilute ammonia, water and dried (Na_2SO_4). The gummy residue left on evaporation of the ether was transferred to a small metal bomb and dissolved in ethanol (8 ml.) with sodium (0·16 g.) and hydrazine hydrate (0·4 ml.). The bomb was sealed and heated at 200–204° for 4·5 hr. The contents of the cooled bomb were then diluted with water and acidified with H_2SO_4. The precipitated solid was collected, washed with water and dissolved in ethanol. Evaporation of this left a residue which was dissolved in a little ether and kept at about 5°, when crystals separated. These (18 mg., m.p. approx. 190°) were collected after some weeks, washed with cold ether and recrystallized from l.p./ethanol, from which small colourless regular prisms (8 mg.) separated. This *deoxypythocholic acid* had m.p. approx. 160°, depending on the rate of heating. (Found: C, 73·6; H, 10·3. $C_{24}H_{40}O_4$ requires C, 73·5; H, 10·2%.) The original ethanol liquors on evaporation left a residue which, with dilute acetic acid, gave crystals (4 mg.) which after two recrystallizations from dilute ethanol gave needles of m.p. 155–159°, not depressed by cholanic acid.

DISCUSSION

Biological. The present results lend considerable support to the view that pythocholic acid will be found to be characteristic of the Boidae, for it has now been found in four different genera of the family. Cholic acid, which forms the chief bile acid in a number of snakes, is also to be found in some Boidae. In *Boa canina*, only about 30% of the 'bile acids' was accounted for, and this suggests that some as yet unidentified bile acid may be present. Snake bile doubtless contains a variable amount of neutral and acidic lipid which may be regarded as in the course of excretion via the liver, and such material will be included in the weight of the 'bile acids'. However, it would be surprising if it constituted as much as 70% of this weight; especially as in two other species, only 30% of the 'bile acids' was not accounted for as pythocholic acid.

It is not of course possible to say whether pythocholic acid should be regarded as a unique bile acid, formed perhaps as the result of a genetic mutation in snakes producing (say) cholic acid, or whether the Boidae have retained pythocholic acid as a legacy from the limbed reptiles from which they may be presumed to have evolved. In considering this question, however, one may notice that the only other lactone-forming bile acids so far said to exist have been isolated from the chelonian reptiles *Amyda japonica* (Yamasaki & Yuuki, 1936) and *Emys orbicularis* (Kim, 1939).

It will be of great interest to compare the chemical constitution, when it is known, of these lactones with that of pythocholic lactone.

If pythocholic acid, is, so to speak, an evolutionary legacy, then perhaps it is being replaced in the Boidae by the generally occurring cholic acid.

Chemical. If the, so far as is known, universal occurrence in the bile acids of a 3α-hydroxyl group is accepted in the present case it may be said with some confidence that pythocholic acid is probably 3α:12:16 (or 15)-trihydroxycholanic acid. Molecular models show that lactone formation is hardly possible with any secondary hydroxyl group other than one at C_{12}, C_{15} or C_{16}. Hence if the evidence for OH groups at C_3 and C_{12} in the lactone is considered strong, the remaining hydroxyl group in pythocholic acid can only be at C_{15} or C_{16}. The chemical and optical evidence, whilst perhaps slightly favouring C_{16}, does not enable us to choose with confidence between these positions, and hence we tentatively formulate pythocholic acid as (I) and its lactone as (IV).

SUMMARY

1. Approximate figures are given for the amounts of cholic acid and pythocholic acid in the bile of seven species of Boidae. The possible significance of the occurrence of these bile acids in boa and python bile is discussed.

2. Pythocholic acid is shown to be a trihydroxycholanic acid. The three hydroxyl groups are secondary and are considered to be probably at C_3, C_{12} and C_{15} or C_{16} in the steroid nucleus. Various derivatives of pythocholic acid and its lactone have been prepared.

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Cholesterol Metabolism

3. CHOLESTEROL METABOLISM IN THE GUINEA PIG AND RABBIT

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Experiments on the metabolism of cholesterol in rats fed chemically defined and animal-cake diets were reported previously (Cook, Polgar & Thomson, 1950). Guinea pigs and rabbits do not consume the synthetic diet readily, but the animal cake is taken well. The experiments reported here are on the metabolism of cholesterol in guinea pigs and rabbits fed a basal ration of animal cake. Balance sheets of the cholesterol have been made, which are compared with those obtained with rats on the animal-cake diet. It is true that numerous balance experiments have been carried out on various animal species (for review see Cook, 1942–3). The general conclusion to be drawn is that when excess cholesterol is fed there is a loss of sterol.

The experiments to be described demonstrate some of the species differences in cholesterol metabolism on basal diets of essentially the same composition. It is to be stressed that the chemical findings are of a preliminary nature. The analytical methods used give, however, gravimetric values for neutral and acidic fractions. Specific methods for dealing with the components of these fractions are being developed.

METHODS

Animals. (*a*) *Guinea pigs.* Male animals about 6 months old and of average weight 400 g. were used. (*b*) *Rabbits.* Male Dutch rabbits about 6 months old and of average weight 1·4 kg. were selected. Both species were obtained locally. Litter mates were used for each set of experiments, the animals being placed in metabolism cages in a room maintained at a temperature of 22°±1°. Other particulars are given by Cook *et al.* (1950).

Diet. The basal diet consisted of rat cake (North-Eastern Agriculture Co-operative Society Ltd., Aberdeen) and contained 5% ether-soluble lipid and 12 mg. sterol/100 g. To this was added olive oil (obtained locally) in the proportion of 200 g. oil/kg. rat cake. When cholesterol was added 20 g. were mixed in the warm oil on a water bath, and when dissolved the mixture was added to the rat cake. The diet of the animals was supplemented with 10 mg. ascorbic acid and 50 g. fresh savoy leaves daily.

Extraction of faecal lipid. The methods used for extraction of lipid and for fractionation have been described by Cook *et al.* (1950). The faeces were not further extracted with ethanol. The results are, however, comparable.

Free fatty acids. Faecal lipids (100 mg.) were dissolved in 20 ml. absolute ethanol and the solution titrated in the cold with 0·1 N-ethanolic KOH. The free acid was calculated as of equivalent 284.

Livers. Pooled liver samples were used and analysed as described by Cook *et al.* (1950).

Carcasses and gut. At the end of an experiment, the carcasses and gut of the guinea pigs were pooled, digested for 6 hr. with 10% (w/v) ethanolic KOH, made up to volume and a sample acidified and extracted with ether. The extracted lipid was then fractionated into unsaponifiable matter and the total sterol estimated. With the rabbits the same procedure was adopted, but the carcasses and gut were analysed separately.

Urine. The total volume of urine collected throughout certain experiments was measured. A sample was strongly acidified with conc. HCl and extracted with 1 vol. of ether divided into three portions. The value given for lipid is the ether-soluble material.

Free and total sterol. Total sterol was determined by the method of Schoenheimer & Sperry (1934) on the lipid from the faeces, carcasses and/or gut, and urine. Free sterol was determined on the faecal lipids.

RESULTS

The duration of experiment, weights of animals, total food, fat and cholesterol ingested are shown in Table 1. For continuity the serial numbers used previously have been continued. The guinea pigs in Exp. IX, S group, did not eat the diet readily and took 14 days longer to consume the same amount of food as did the control animals. The increase in weight of the cholesterol-fed animals was only slight as compared with that of the control animals. In Exp. X approximately the same amount of food was consumed by both series of animals in the same time, but the animals in both series lost weight, the effect being most marked in Exp. X, S group.

In the rabbit experiments three animals were used initially in both groups, but at the end of 58 days one of the cholesterol-fed animals died. The experiment was continued with two animals in each group. During the first period (XI) the cholesterol-fed animals took the diet well and increased in weight, but during the second period (XII, S group) the intakes of food were poor and the animals steadily lost weight. The bodies of the cholesterol-fed animal which died after 58 days and of the third control animal were retained for analysis.

Table 1. *Duration of experiment, weights of animals, total food, fat and added cholesterol ingested*

(In this and subsequent tables C = control group, S = group fed diet plus cholesterol.)

Animals	Exp. no.	No. of animals	Increase or decrease in wt. during experiment (g.)	Duration of experiment (days)	Total food (g.)	Fat (g.)	Added cholesterol (g.)
Guinea pigs	IX C	2	+ 247	30	1060	177	0
	IX S	2	+ 26	44	1070	175	17·6
	X C	2	− 127	34	1230	205	0
	X S	2	− 223	34	1105	181	18·1
Rabbits	XI C	3	+2170	58	6080	1015	0
	XI S	3	+2271	58	6680	1095	109·5
	XII C	2	+ 220	18	1650	275	0
	XII S	2	− 720	19	450	74	7·4

Table 2. *Faecal excretion of guinea pigs and rabbits*

(In this and subsequent tables, —, means 'not determined'.)

Animals	Exp. no.	Wt. faeces (g.)	Ether-soluble lipid (g.)	Unsaponifiable matter (U.M.) (g.)	Total sterol (g.)	Total acids (g.)	Light petroleum-insoluble acids (g.)
Guinea pigs	IX C	236	36·3	9·6	0·8	20·9	—
	IX S	367	76·8	19·3	5·2	48·5	—
	X C	315	53·5	5·8	1·2	40·5	0·22
	X S	328	70·3	17·5	9·4	44·2	0·34
Rabbits	XI C	1289	80·4	14·9	4·1	57·5	6·7
	XI S	1335	134·0	63·1	25·2	63·7	3·0
	XII C	302	15·5	2·8	0·9	9·7	—
	XII S	141	14·6	4·6	2·4	7·1	—

In Table 2 are given the values for the excretion of faecal lipids. In these experiments the ether-soluble lipid only was investigated, no further extraction with ethanol being carried out. The data, therefore, with respect to the total acids and particularly the light petroleum-insoluble acids are not complete. They are, however, directly comparable.

The unsaponifiable matter (U.M.) and total sterol were increased in amount in both species after cholesterol feeding. With the guinea pigs in Exp. IX there was a large increase in the total acids excreted after cholesterol feeding, but this difference was not so marked in Exp. X. It is possible that the longer duration of Exp. IX, S group, affected the absorption of fat. Guinea pigs do not take readily to a diet rich in fat, and the experiments could not be continued for as long as the rabbit experiments. With the rabbits (Exp. XI) the increase in excretion of total acids after cholesterol feeding was slight compared with the amount of cholesterol fed, and in Exp. XII there was a decrease in the excretion of acids.

Determinations of the light petroleum-insoluble acids were not done in all experiments but with the guinea pigs there was a slight increase after cholesterol feeding. With the rabbits a large amount was present in the faecal acids of the control animals, but the amount was decreased after cholesterol feeding.

The species difference in excretion is better illustrated by the values for the faecal components as percentages of the total ether-soluble lipid. These are shown in Table 3 where the values for the faecal lipids of rats (Exp. VI, Cook *et al.* 1950) have been added for comparison. In all species after the feeding of cholesterol the U.M. as percentage of lipid increased in amount, as did the total sterol. The sterol as percentage of U.M. was greatest with the rat and least with the rabbit. The percentage of ester sterol was lowest with the guinea pigs and highest with the rat. Total fatty acids were of the same order in the faeces of the control animals but the percentage fell after cholesterol feeding due to dilution by U.M. An interesting feature is the high percentage of free fatty acids excreted by the guinea pig. The light petroleum-insoluble acids formed a low percentage of the acids in both control and cholesterol-fed guinea pigs, but there was an increase with the latter animals. In the rabbit these acids formed a high percentage of the faecal acids of the control animals, but after cholesterol feeding the percentage fell. The high proportion of light petroleum-insoluble acids and its increase after cholesterol feeding in the rats has already been noted.

The urinary excretion values for certain experiments are given in Table 4 and are incomplete. They do demonstrate that there was a slight additional excretion of lipid in the urine of the guinea pigs, and

Table 3. *Distribution of faecal lipids*

(Values as percentage of ether-soluble lipid, except where stated otherwise.)

	Guinea pigs (Exp. X)		Rabbits (Exp. XI)		Rats (Exp. VI)	
	C	S	C	S	C	S
U.M.	11	25	19	47	19	55
Total sterol	2	13	5	19	9	37
Ester sterol	0·4	2	2	4	4	9
Total fatty acids	80	63	72	48	61	41
Free fatty acids	76	62	50	29	30	26
Light petroleum-insoluble acids	0·4	0·5	8	2	6	7
Sterol as % U.M.	18	52	26	40	47	67
Ester as % total sterol	20	15	40	21	44	24

Table 4. *Urinary excretion*

Animals	Exp. no.	Volume (l.)	Total lipid (g.)	Total sterol (mg.)
Guinea pigs	IX C	1·46	0·7	—
	IX S	1·12	1·2	—
Rabbits	XI C	18·9	20·9	80
	XI S	17·6	33·6	210

Table 5. *Values for livers*

Animals	Exp. no.	No. of livers	Wt. (g.)	Livers (as % body wt.)	Lipid (as % fresh liver wt.)	U.M. (g.)	U.M. (as % lipid)	Total sterol (g.)	Total sterol (as % lipid)
Guinea pigs	IX C	2	35·4	4·1	7·1	0·15	6·0	0·1	4·0
	IX S	2	76·7	10·2	18·8	2·25	15·6	1·6	11·0
	X C	2	44·8	4·2	5·9	0·3	10·0	—	—
	X S	2	56·5	6·9	15·2	1·7	19·8	1·2	13·4
Rabbits	XI C	3	138·6	3·4	6·3	0·8	10·3	0·6	6·9
	XI S	1*	90·3	7·7	19·7	7·2	40·4	5·3	29·8
	XII S	2†	247·3	8·6	20·4	21·9	43·5	16·7	33·2
Rats	VIII C	2	19·6	2·9	7·9	0·1	7·0	0·1	5·7
	VIII S	2	40·5	5·5	17·3	2·1	30·6	1·8	25·0

* 58 days. † 77 days.

that with the rabbits, although the amount of lipid was increased after cholesterol feeding, the increased excretion of sterol was slight. The nature of the urinary lipids in guinea pigs and rabbits will be investigated later.

In Table 5 are given the values for the liver lipids. In Exp. IX and X two livers were pooled and analysed. In Exp. XI, C group, three livers were pooled, but the value for Exp. XI, S group, is for the liver of the animal which died after 58 days. In Exp. XII, S group, two livers were pooled. The large increase in size of the guinea pig liver after cholesterol feeding, particularly in Exp. IX, S group, is noteworthy. The percentage of lipid increased two- to three-fold, and the U.M. and sterol constituted as mean values 18 and 12 % respectively of the lipid.

In the rabbit there was an increase in size of the liver and an increase in the percentage of lipid, but the most striking increase as compared with the guinea pig was in the amount of U.M. and of sterol (42 and 32 % respectively of the lipid). The values for rat livers are given for comparison and appear to be intermediate between those for the guinea pig and rabbit.

Analysis of the carcasses and gut are given in Table 6. With the guinea pigs the two parts were analysed together and the results shown are for two pooled animals in each experiment. With the rabbit, carcasses and gut were dealt with separately. For the control group three animals were pooled. In the cholesterol-fed group, the carcass and gut of the animal that had died after 58 days was pooled and analysed with the two animals in Exp. XII. The amount of lipid in the carcasses and/or gut of the animals fed cholesterol was much less than with the control animals. This confirmed the visual observation that the cholesterol-fed animals had poor fat depots. This effect of cholesterol on the depot fats of rats is seen only in experiments of duration greater than 60 days (Cook, 1936, 1937). The amount of

Table 6. *Analyses of carcasses and gut*

(All results in g.)

	Exp. no.	Carcass Wt.	Carcass Lipid	Carcass U.M.	Carcass Sterol	Gut Lipid	Gut U.M.	Gut Sterol
Guinea pigs	IX C	846	41·7	1·1	0·7	Included in carcass		
	IX S	677	29·0	2·2	1·4	Included in carcass		
	X C	950	46·8	1·4	—	Included in carcass		
	X S	720	24·6	2·7	1·4	Included in carcass		
Rabbits	C	3160	388·0	7·4	3·8	42·5	2·0	1·0
	S	2772	181·3	15·6	11·6	33·2	4·3	2·1

Table 7. *Unsaponifiable matter* (U.M.) *balance of the various species*

(The differences in U.M. content of the ether-extractable lipid of the cholesterol-fed and control animals for faeces, livers, carcasses and/or gut are given. The sum *A* represents total U.M. accounted for and *B* the amount of ingested cholesterol. *B* – *A* is the amount of cholesterol (as g.) unaccounted for, this value being shown also as %. The values for the increase in total faecal acids and light petroleum-insoluble acids are given in the last two lines. The values for the rats are from Cook *et al.* (1950).)

	Guinea pigs IX	Guinea pigs X	Rabbits XII	Rats VI	Rats VIII
Exps.					
Difference (g.) of U.M. in Faeces	9·7	11·7	50·0	13·9	16·8
Difference (g.) of U.M. in Livers	2·1	1·4	28·3	1·0	2·1
Difference (g.) of U.M. in Carcasses	1·1	1·3	8·2	—	—
Difference (g.) of U.M. in Gut	—	—	2·3	—	—
Total, *A* (g.)	12·9	14·4	88·8	14·9	18·9
Cholesterol fed, *B* (g.)	17·6	18·1	116·9	18·9	22·1
Cholesterol unaccounted for (*B* – *A*) (g.)	4·7	3·7	28·1	4·0	3·2
Cholesterol unaccounted for (as % of amount fed)	27	20	24	21	14
Increase in total faecal fatty acids (g.)	27·6	3·7	3·6*	5·6	9·1
Increase in light petroleum-insoluble acids (g.)	—	0·1	−3·7*	1·5	1·0

* For Exp. XI.

Table 8. *Total sterol balance of the various species*

(The differences in sterol content of the ether-extractable lipid of the cholesterol-fed and control animals are given.)

	Guinea pigs IX	Guinea pigs X	Rabbits XII	Rats VI
Exps.				
Difference (g.) of total sterol in Faeces	4·4	8·2	22·6	9·7
Difference (g.) of total sterol in Livers	1·5	1·2	21·4	0·9
Difference (g.) of total sterol in Carcasses	0·7	0·7	7·8	—
Difference (g.) of total sterol in Gut	—	—	2·3	—
Total, *A* (g.)	6·6	10·1	54·1	10·6
Cholesterol fed, *B* (g.)	17·6	18·1	116·9	18·9
Cholesterol unaccounted for (*B* – *A*) (g.)	11·0	8·0	62·8	8·3
Cholesterol unaccounted for (as % of amount fed)	63	44	54	44

U.M. and of sterol was increased slightly in the carcasses and gut of the guinea pigs, but large amounts were present in the cholesterol-fed rabbits. There would appear to be a generalized deposition of cholesterol in this animal (Aylward & Stott, 1937).

In the rat the deposition of cholesterol in the carcass was slight and may be neglected in constructing a balance sheet (Cook, 1937).

From the results given in Tables 2, 5, and 6 it is possible to construct balance sheets of the sterol metabolism both on the differences in U.M. content in cholesterol-fed and control animals, and on the difference in sterol content. The values for the urinary excretion have been omitted as they are incomplete, but the error so introduced is slight. Balance sheets based on the U.M. are shown in Table 7 where values for certain experiments on the rat (Cook *et al.* 1950) are given. The results for the guinea pigs show that there is a mean loss of 24 % of the ingested cholesterol. In Exp. IX there was a large increase in the excretion of total fatty acids, but in Exp. X the increase was equal in amount to the missing cholesterol. Determinations of the light petroleum-insoluble acids were not carried out in Exp. IX, but the increase in Exp. X was slight. With rabbits there is a 24 % loss of cholesterol but only a

slight increase in the excretion of total faecal acids. The difference between the light petroleum-insoluble acids is actually negative, i.e. more acids are produced by the control animals.

The results with rats are shown for comparison and give a mean loss of 18% cholesterol. The increase in total faecal acid and its correspondence with the missing cholesterol have been commented on before. The rat shows the greatest production of light petroleum-insoluble acids on the cholesterol-containing diet.

Balance sheets from certain experiments based on the differences in sterol content are given in Table 8. With guinea pigs the mean loss of cholesterol as a difference between the cholesterol-fed and control animals is 54% of that ingested, with rabbits 54% and the value for the rat is 44%. These values are greater than those obtained for U.M. difference, and indicate that part of the cholesterol is converted into compounds which appear in the U.M. (cf. Page & Menschick, 1932; Bossert, Page & Menschick, 1934).

It is interesting to observe that, whether based on loss of U.M. or of sterol, there would appear to be a greater percentage loss of cholesterol in the guinea pig and rabbit than there is the rat. The rat, however, produces excess faecal fatty acids which correspond to the loss of cholesterol and these acids contain a high percentage of light petroleum-insoluble acids.

DISCUSSION

The reactions of the rat, guinea pig and rabbit to the presence of large amounts of cholesterol in the diet are very different. Rats can apparently tolerate large amounts of dietary cholesterol, and although they develop 'fatty' livers, suffer little inconvenience. The guinea pigs and rabbits are affected very adversely. The guinea pigs lose weight and develop greatly enlarged 'fatty' livers and spleens. The rabbits for a time grow and maintain weight, but later there is a loss of appetite and weight.

The ingestion of cholesterol causes a generalized lipid deposition including deposition of cholesterol and its esters in the intima of the aorta, giving rise to the pathological picture of atheroma. The pathological changes observed in rats, guinea pigs and rabbits are described in detail by Cook & McCullagh (1939).

Okey & Greaves (1939) also described the development of a marked anaemia in guinea pigs. Experiments with other animals are reviewed by Cook (1942–3). The question arises as to how these different reactions are related to the metabolism of cholesterol.

The absorption of fat (olive oil) and of cholesterol in the rat, guinea pig and rabbit is described in detail by Cook & Thomson (1950). On the animal rat-cake diet containing 16·6% fat the following absorptions of fat (as %) were observed: rat, 92; guinea pig, 77 and rabbit 94. With fat and 1·6% cholesterol the cholesterol absorptions (measured as U.M.) were (as %) rat 34, guinea pig 47, and rabbit 77. The absorption of fat by the guinea pig is thus less than with the other species, but the order of cholesterol absorption is rat, guinea pig, rabbit. This order is reflected in the levels of plasma cholesterol obtained which in the rabbit may reach values as high as 2000 mg./100 ml. (Cook & Thomson, 1950). One possibility, therefore, is that the species difference is due to the fact that absorption occurs more readily in some species than others (cf. rat and rabbit).

The relation of the excretion of faecal acids to the absorption process is not clear, but while there is a considerable excretion of excess acids in the cholesterol-fed guinea pigs and rats, the increase was slight with the rabbits in Exp. XI, and in Exp. XII there was a decrease. The overall excretion of acids corresponded to 15% of the missing cholesterol. It is probable that there is some connexion between the absorption of cholesterol and the excretion of acids. Experiments on the rat show that cholesterol hinders the absorption of fat and that in cholesterol-fed animals there is an increased excretion of ordinary fatty acids (Edwards & Cook, 1951). Acetic acid and other volatile acids were also increased in amount (Thomson, 1951).

That cholesterol is broken down is certain and in the rat part of the degradation products appear in the light petroleum-insoluble fraction. The results given here show that the cholesterol-fed guinea pigs have a slightly increased production of light petroleum-insoluble acids, but the rabbit is different in that greater amounts are produced with the control animals than with those fed cholesterol.

The experiments of Schoenheimer & Breusch (1933) demonstrated that in mice fed normal diets there was a balance between cholesterol synthesis and destruction. This may well be true of other species, but when excess cholesterol is fed, as in the experiments described here, degradation exceeds synthesis. The pathways of degradation are numerous and may result in the production of neutral products such as coprostanol, which would appear in the unsaponifiable matter fraction, or in the production of acidic compounds. The complete identification of the products of metabolism will be a lengthy procedure.

One conclusion at least is clear from the experiments described here, which is that there is a marked species difference in response to the inclusion of excess cholesterol in the diet.

SUMMARY

1. Guinea pigs and rabbits were fed a basal diet of animal cake and 16·6% olive oil with and without 1·6% cholesterol. The faecal excretion of lipid and its deposition in the tissues were studied.

2. The ether-soluble faecal lipids were fractionated into unsaponifiable matter, total sterol, total acids and light petroleum-insoluble acids (Table 2). The distribution of the faecal lipids was determined (Table 3).

3. The deposition of unsaponifiable matter and sterol in the livers, carcasses and gut was determined (Tables 5 and 6).

4. Balance sheets of the cholesterol metabolism have been made both on differences in unsaponifiable matter (Table 7) and the differences in sterol (Table 8) between cholesterol-fed and control animals. Calculated as unsaponifiable matter an average of 24% of the cholesterol ingested was metabolized in guinea pigs and in rabbits (one experiment) 24%. As sterol the guinea pigs and rabbits metabolized 54%. For rats these values are, unsaponifiable matter, 18% and sterol 44%.

5. In guinea pigs there was an increased excretion of total faecal fatty acids in the cholesterol-fed animals. In the rabbit this increase is slight.

The light petroleum-insoluble acids were slightly increased in the faeces of cholesterol-fed guinea pigs, but in the rabbit more light petroleum-insoluble acids are present in the faeces of control animals than there are in those fed cholesterol.

6. The significance of these findings in relation to the pathological lesions produced in these different species by feeding excess cholesterol in this diet is discussed.

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Vitamin A in the Kidney of the Rat

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Johnson & Baumann (1947*a*, *b*) reported that when rats were kept on a diet deficient in vitamin A with supplements of 35 i.u. of carotene daily, or of small doses of preformed vitamin A, more vitamin A was accumulated in their kidneys than in their livers. With higher doses, however, the usual predominance of the liver as the main site of storage was asserted.

In a preliminary communication we have reported experiments which confirmed this interesting observation (Eden & Moore, 1950). The purpose of the present paper is to present our results in detail. Since Moore & Sharman (1950) have recently found that the distribution of vitamin A between the liver and kidneys is influenced by sex our results on male and female animals will be considered separately.

EXPERIMENTAL

In most of our experiments groups of young piebald rats, weaned from mothers receiving an adequate mixed diet, were given for 4–7 weeks a diet of casein 20%, sugar 45%, dried brewer's yeast 10%, minerals 5% and either lard (groups 1, 2, 3, 6 and 7 in Table 1) or cod liver oil 20% (groups 5 and 8). The diet with lard, and without supplements of vitamin A, caused the disappearance of vitamin A from both the liver and kidneys within 5 weeks. Groups 1–3 received daily supplements of 20 i.u. of synthetic vitamin A acetate, which was kindly supplied by Roche Products Ltd. The animals of groups 6 and 7 received only a single dose of 2000 i.u. of vitamin A acetate 48 hr. before they were killed. The cod liver oil supplied to groups 5 and 8 was of a high class medicinal grade containing about 800 i.u./g. of vitamin A, and was mixed freshly each day with the solid components of the diet. The rats in group 4 were adult males, about 1 year old, which had been used for breeding purposes; their diet had been made up of bread, milk, meat and vegetables, with occasional doses of cod liver oil.

It may be mentioned that the rats in groups 1, 2, 5, 6 and 8 received a diet lacking in vitamin E. The animals in groups 3 and 7 were each dosed with 2 mg. of (±)-α-tocopheryl acetate weekly, while those in group 4 had derived adequate supplies from their mixed diet. Although it is known that vitamin E may influence the storage of vitamin A in some

Table 1. *The accumulation of vitamin A in the livers and kidneys of rats at various levels of intake*

Sex	Group no.	Rat no.	Weight Initial (g.)	Weight Final (g.)	Vitamin E	Daily intake vitamin A (i.u.)	Time given vitamin A (days)	Form of vitamin A given	Vitamin A (i.u.) total in Liver	Vitamin A (i.u.) total in Kidney	Vitamin A (i.u./g.) in Liver	Vitamin A (i.u./g.) in Kidney
Male	1	1	36	126	–	20	28	Acetate	71	103	10·4	70
		2	40	130	–	20	28	Acetate	71	96	9·8	68
		3	50	145	–	20	28	Acetate	33	83	4·3	59
Male	2	4	51	200	–	20	34	Acetate	28	75	2·6	40
		5	52	185	–	20	34	Acetate	83	59	9·3	37
		6	55	212	–	20	34	Acetate	26	69	2·3	35
Male	3	7	38	138	+	20	28	Acetate	330	91	42	67
		8	38	145	+	20	28	Acetate	93	200	11	140
		9	33	121	+	20	28	Acetate	80	103	10	80
							Means for groups 1–3 combined		**91**	**98**	**11**	**66**
Male	4	10	Final		+	.	Approx. 365	Mixed diet	2,700	28	250	16
		11	weights		+	.	Approx. 365	Mixed diet	3,700	36	300	20
		12	≃ 350 g.		+	.	Approx. 365	Mixed diet	4,000	32	320	16
		13			+	.	Approx. 365	Mixed diet	3,200	38	310	20
		14			+	.	Approx. 365	Mixed diet	1,600	33	110	17
		15			+	.	Approx. 365	Mixed diet	3,120	29	320	13
		16			+	.	Approx. 365	Mixed diet	2,300	52	210	27
		17			+	.	Approx. 365	Mixed diet	4,600	15	400	9
								Means for group 4	**3,200**	**33**	**280**	**17**
Male	5	18	45	218	–	Approx. 2,000	50	Cod liver oil	16,000	570	1,400	190
		19	38	163	–	Approx. 2,000	52	Cod liver oil	26,000	520	4,100	330
		20	33	154	–	Approx. 2,000	52	Cod liver oil	57,000	180	6,700	100
		21	43	166	–	Approx. 2,000	52	Cod liver oil	7,900	17	1,200	8
								Means for group 5*	**27,000**	**320**	**3,400**	**160**
Female	6	22		122	–	Approx. 2,000	1	Acetate	360	15	57	11
		23		149	–	Approx. 2,000	1	Acetate	320	35	47	22
		24		154	–	Approx. 2,000	1	Acetate	360	41	46	23
	7	25		127	+	Approx. 2,000	1	Acetate	270	10	45	8·5
		26		140	+	Approx. 2,000	1	Acetate	240	17	34	11
		27		120	+	Approx. 2,000	1	Acetate	460	24	69	16
								Means for groups 6 and 7	**340**	**24**	**50**	**15**
Female	8	28	47	112	–	Approx. 2,000	29	Cod liver oil	3,900	16	600	10
		29	38	104	–	Approx. 2,000	29	Cod liver oil	6,000	14	840	10
		30	40	97	–	Approx. 2,000	29	Cod liver oil	7,500	6	1,200	6
								Means for group 8	**5,800**	**12**	**880**	**8·7**

* In our preliminary communication (Eden & Moore, 1950) the means for the kidneys in this group were miscalculated.

circumstances (Moore, 1940) there was no plain evidence of this effect being exercised in the present experiments, which were too short to cause an acute deficiency of vitamin E. In taking averages, therefore, the values for the groups with and without vitamin E at the same levels of dosing with vitamin A have been combined.

In most instances the animals were killed for the estimation of vitamin A in their livers and kidneys, but those in group 5 died spontaneously or accidentally. The vitamin was extracted from the tissues after they had been digested with alkali according to the method of Davies (1933), and was measured by the antimony trichloride method applied with a photoelectric absorptiometer. In Exp. 4 the rats nos. 10–13 were killed with coal gas and nos. 14–17 with $CHCl_3$, but the mode of killing has no obvious effect on the distribution of the vitamin. Two of the animals in group 5 died from profuse bleeding from minor accidental wounds in their paws, while another had subcutaneous and intramuscular haemorrhage in the inguinal region. These injuries were presumably caused or exacerbated by the heavy intake of cod liver oil. The results obtained are given in Table 1.

Vitamin A in other tissues. Estimations of vitamin A were not made systematically in tissues other than the liver and kidney. In specimens of lungs, suprarenals and retinas collected from different groups, however, the total amounts of vitamin A were invariably much smaller than in the kidneys, and were seldom sufficient, except in the lungs, to produce blue colours discernible to the eye in the antimony trichloride reaction.

DISCUSSION

Males. From Table 1 it will be seen that in the nine male rats of groups 1–3 which were given 20 i.u. of vitamin A daily, the concentration of the vitamin was invariably greater in the kidney than in the liver, with means of 66 and 11 i.u./g., respectively. In seven instances the total amounts of vitamin A were greater in the kidneys than in the liver, with means of 98 and 91 i.u. In the eight rats of group 4, which had received an adequate mixed diet for about a year, the normal relationship between the concentrations in the kidneys and liver was found, with values of 17 and 280 i.u., respectively. In the four rats of group 5, which received large amounts of cod liver oil for 7 weeks, the mean concentrations were 160 i.u./g. in the kidneys, and 3400 i.u. in the liver. These results confirm the statement of Johnson & Baumann (1947*a*, *b*) that the kidney is the main site of storage of vitamin A at low levels of dosing. The changes in the concentration of vitamin A in the kidneys in response to increased intakes of vitamin A, however, were surprisingly inconsistent. From the limited number of results which are at present available it would appear that the amounts of vitamin A in the kidneys are high at rather low and very heavy levels of intake, and lower at intermediate intakes. The complicating factor that the rats in group 5 died, whereas those in the other groups were killed, must not however be overlooked. The effect of very heavy doses of vitamin A in raising its level in the kidneys has already been reported by Davies & Moore (1934).

Females. In the female rats of groups 6–8 the intakes of vitamin A were too high to allow the concentrations reached in the kidneys to exceed those found in the liver. The results may be interesting, however, as further evidence of a lack of simple proportionality between the concentrations in the liver and kidneys. Thus in the combined groups 6 and 7 the kidney and livers averaged 15 and 50 i.u./g., respectively. Although in group 8 the mean concentration of 880 i.u./g. in the liver was nearly twenty times higher, the level in kidneys was only 8·7 i.u./g.

SUMMARY

1. The priority of the kidneys over the liver in accumulating vitamin A at low levels of intake was confirmed in young male piebald rats.

2. The usual predominance of the liver was asserted at higher levels of intake. In both male and female animals no simple relationship could be found between the concentrations in the liver and kidneys.

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*allo*Pregnane-3*a*:20*a*-diol in the Urine of Pregnant Women

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*allo*Pregnane-3α:20α-diol was first isolated from human pregnancy urine by Hartmann & Locher (1935). The method employed consisted essentially of separating pregnane-3α:20α-diol diacetate from an acetylated crude 'pregnanediol' fraction as completely as possible by crystallization from ethanol, saponifying the mother liquor, and crystallizing from ethanol. A similar method was employed later by Beall (1937). A method for the estimation of the amount of *allo*pregnane-3α:20α-diol in urine, involving oxidation of the crude 'pregnanediol' fraction and separation of the mixed diketones by fractional crystallization of their disemicarbazones, was suggested by Marker, Kamm, Crooks, Oakwood, Lawson & Wittle (1937), and later Marker, Wittle & Lawson (1938) published figures indicating that the ratio of pregnane-3α:20α-diol to *allo*pregnane-3α:20α-diol in the urine of pregnant women is about 2:1.

In the course of other work in this laboratory the chance isolation of *allo*pregnane-3α:20α-diol in a state of high purity by a relatively simple procedure suggested the possibility of developing a method which would be more satisfactory for semi-quantitative work than the isolation process of Hartmann & Locher or the somewhat drastic procedure of Marker *et al.* In the present paper such a method is described and a re-examination of the problem of the ratio of pregnane-3α:20α-diol to *allo*pregnane-3α:20α-diol in the urine of pregnant women is reported. Some preliminary observations upon the conjugated form of *allo*pregnane-3α:20α-diol in human pregnancy urine are also reported.

EXPERIMENTAL

Methods. All melting points were determined on an improvised hot-stage apparatus of the Kofler type (Klyne & Rankeillor, 1947). The apparatus was calibrated with pure substances of known melting point. The melting points recorded are therefore corrected.

Al_2O_3 used for chromatography was obtained from Peter Spence and Sons and was activated before use to activity II, as determined by the method of Brockmann & Schodder (1941), by heating *in vacuo* at 100°.

All solvents used were carefully dried and redistilled. Samples for analysis were dried over P_2O_5 *in vacuo* for 6 hr. at 80° unless stated otherwise.

Initial isolation of allo*pregnane-3α:20α-diol*

The starting material was the 95% acetone-soluble fraction from the preparation of 'sodium pregnanediol glucuronidate' by the method of Venning & Browne (1936) from about 450 l. of the urine of pregnant women. (The term 'sodium pregnanediol glucuronidate' is here used to denote the mixture of sodium glucuronidates which can be obtained from human pregnancy urine by the method of Venning & Browne.) After hydrolysis with HCl the ether-soluble neutral fraction (28·6 g.) was prepared in the usual way and separated into ketonic and non-ketonic fractions by means of Girard's reagent T (Girard & Sandulesco, 1936). The non-ketonic fraction (15·4 g.) was dissolved in benzene and poured on a column containing 450 g. Al_2O_3. The column was then eluted successively with 2600 ml. benzene, 3000 ml. ether and 4000 ml. acetone. The acetone eluate on evaporation to dryness yielded a brown crystalline mass weighing 7·5 g. This material on recrystallization from methanol yielded 220 mg. of colourless stout crystalline rods melting at 245–245·5°. On admixture with authentic pregnane-3α:20α-diol the melting point was 214–216°. (Found: C, 78·7, 78·7; H, 11·1, 10·9. Calc. for $C_{21}H_{36}O_2$: C, 78·8; H, 11·2%.)

An acetate of the substance was prepared in the usual manner with acetic anhydride and pyridine. After recrystallization from methanol a product melting at 141·5° was obtained. (Found: C, 74·6; H, 9·9. Calc. for $C_{25}H_{40}O_4$: C, 74·3; H, 9·9%.) $[\alpha]_D^{19°} + 15·5° \pm 0·1°$ in ethanol (*c*, 0·5608).

A portion of the original substance was oxidized at room temperature overnight with CrO_3 in 90% aqueous acetic acid. The product after crystallizing once from aqueous ethanol and once from acetone melted at 200–202° with preliminary softening at 195°.

On treatment with semicarbazide hydrochloride in aqueous pyridine the oxidation product yielded a semicarbazone which after washing with hot ethanol did not melt below 300°. The sample for analysis was dried for 8 hr. at 130°. (Found: N, 19·6. Calc. for $C_{23}H_{38}O_2N_6$: N, 19·6%.)

A comparison of the properties of the substance isolated and its derivatives with those of *allo*pregnane-3α:20α-diol and its corresponding derivatives which are reported in the literature provided convincing proof that the substance was *allo*pregnane-3α:20α-diol (see Table 1).

Solubilities of pregnane-3α:20α-diol and allo*pregnane-3α:20α-diol*

Mixtures consisting largely of pregnane-3α:20α-diol and *allo*pregnane-3α:20α-diol can be obtained from the neutral ether-soluble fraction of acid-hydrolysed human pregnancy urine by the ethanol-

Table 1. *Comparison of substance isolated with* allo*pregnane-3α:20α-diol*

Substance isolated			*allo*Pregnane-3α:20α-diol		
Original substance	m.p.	245–245·5°	Diol	m.p.	248–248·5° (*a*)
	Analysis (found)	C, 78·7% H, 11·0%		Analysis (calc.)	C, 78·8% H, 11·2%
Acetylated product	m.p.	141·5–142·5°	Diacetate	m.p.	141·5–142·5° (*a*)
	$[\alpha]_D^{19°}$ (ethanol)	+15·5°		$[\alpha]_D^{19°}$ (benzene)	+18·8° (*a*)
				$[\alpha]_D^{24°}$ (ethanol)	+16·0° (*c*)
	Analysis (found)	C, 74·6% H, 9·9%		Analysis (calc.)	C, 74·3% H, 9·9%
Oxidation product	m.p.	200–202°	Diketone	m.p.	204–204·5° (*a*)
Semicarbazone of oxidation product	m.p. Analysis (found)	>300° N, 19·6%	Disemicarbazone of diketone	m.p. Analysis (calc.)	>325 (*b*) N, 19·6%

(*a*) Hartmann & Locher (1935). (*b*) Marker *et al.* (1937). (*c*) Hirschmann & Hirschmann (1950).

Table 2. *Solubilities of pregnane-3α:20α-diol and* allo*pregnane-3α:20α-diol*

(The values shown are in g./100 ml.; each is the mean of duplicate determinations.)

	Pregnane-3α:20α-diol			*allo*Pregnane-3α:20α-diol		
	At b.p. of solvent	At 20°	At 0°	At b.p. of solvent	At 20°	At 0°
Methanol	1·8	—	0·33	0·45	—	0·07
Ethanol	2·6	—	0·50	1·0	—	0·18
Benzene	0·55	0·06	—	0·28	0·06	—
Acetone	—	—	0·03	—	—	0·02
Chloroform	—	0·23	—	—	0·13	—

water precipitation process of Astwood & Jones (1941), or by chromatography on Al_2O_3. As a first step towards developing a simple procedure for the isolation in high yield of *allo*pregnane-3α:20α-diol from such mixtures, the approximate solubilities of both diols were determined at various temperatures, in various solvents with the aid of the apparatus described by Klyne & Bell (1946). The results of the determinations are shown in Table 2.

Separation of a mixture of pregnane-3α:20α-diol and allo*pregnane-3α:20α-diol*

The fact that the solubilities of the two diols in benzene at 20° are about the same, whereas pregnane-3α:20α-diol is about five times more soluble than *allo*pregnane-3α:20α-diol in methanol at 0° indicated that it should be possible to isolate the latter in high yield from a pure binary mixture by leaching with a suitable volume of benzene and crystallizing the benzene-soluble fraction at 0° from methanol. To test this possibility the following experiment was performed.

Pregnane-3α:20α-diol. (700 mg., m.p. 236–237°) was refluxed for 1 hr. with 100 ml. benzene. After standing overnight at room temperature the insoluble material was filtered off and dried, and the benzene filtrate evaporated to dryness. The benzene-soluble material was dissolved in 7 ml. methanol and the mixture set aside in the refrigerator to crystallize. After 3 days fine white needles melting at 237° were deposited. The melting point was unchanged on admixture with authentic pregnane-3α:20α-diol. Since the benzene-soluble fraction yielded only pregnane-3α:20α-diol on crystallization from methanol, it was assumed with some confidence that the benzene-insoluble material was pregnane-3α:20α-diol uncontaminated with any traces of *allo*pregnane-3α:20α-diol.

This material (670 mg.) and *allo*pregnane-3α:20α-diol (13·5 mg.) were dissolved in ethanol and the solution evaporated to dryness. The residue was refluxed for 2 hr. with 100 ml. benzene and after standing overnight the mixture was filtered. The benzene-soluble material obtained by evaporation of the filtrate was dissolved in 5 ml. methanol and the solution allowed to stand for 7 days. The thick crystalline rods which separated (7·0 mg.) melted at 242–243° and did not depress the melting point of authentic *allo*pregnane-3α:20α-diol.

A further 1·5 mg. of *allo*pregnane-3α:20α-diol (m.p. 238–241°) was obtained by treating the benzene-insoluble material again with a further 100 ml. benzene.

From the mixture of 670 mg. of pregnane-3α:20α-diol and 13·5 mg. of *allo*pregnane-3α:20α-diol, a total of 8·5 mg. (63%) of the latter was recovered.

Recovery of allo*pregnane-3α:20α-diol added to the neutral ether-soluble fraction of human urine*

Although the separation of *allo*pregnane-3α:20α-diol in high yield from a pure binary mixture of both diols had been accomplished, it could not be assumed that the procedure would necessarily give such satisfactory results when applied to fractions obtained from human urine. To test this point a recovery experiment was carried out using the

neutral ether-soluble fraction from the acid-hydrolysed urine obtained from a post-menopausal woman receiving 100 mg. progesterone/day by intramuscular injection for 15 days.

All the urine excreted during the 15-day period of treatment was collected and hydrolysed by boiling for 10 min. after the addition of one-tenth of its volume of concentrated HCl. The neutral ether-soluble fraction (1·24 g.) was obtained in the usual way and after solution in ethanol was divided into two equal portions. To one of these portions 10 mg. of *allo*pregnane-3α:20α-diol was added. Both portions were then separately and simultaneously treated as follows: After evaporation of the ethanol the residue was dissolved in 100 ml. benzene and the solution poured on to a column containing 20 g. Al_2O_3. The column was eluted in succession with 1000 ml. benzene, 1000 ml. ether and 350 ml. 15% (v/v) methanol in acetone.

The residue obtained by the evaporation of the methanol-acetone eluate was refluxed with 25 ml. benzene, and after standing overnight at room temperature the mixture was filtered. The benzene filtrate was then poured on to a column containing 5 g. Al_2O_3. The column was eluted successively with 200 ml. benzene, 100 ml. 50% (v/v) ether-benzene, 100 ml. ether, and 75 ml. 5% (v/v) methanol in ether. The methanol-ether eluate was evaporated to dryness and the semi-crystalline residue crystallized twice from methanol.

The portion of the starting material to which no *allo*pregnane-3α:20α-diol had been added yielded 1·0 mg. of thick crystalline rods melting at 238–244° which did not significantly depress the melting point of authentic *allo*pregnane-3α:20α-diol. The portion to which 10 mg. of *allo*pregnane-3α:20α-diol had been added yielded 8·0 mg. of similarly shaped crystals melting at 239–242° which also did not depress the melting point of the authentic compound. Thus 70% of the *allo*pregnane-3α:20α-diol added to the neutral ether-soluble urinary fraction was recovered.

The isolation of *allo*pregnane-3α:20α-diol from the control urine in this experiment does not constitute conclusive evidence that this substance is a metabolic reduction production of progesterone. Urine collected from the same woman before or after the progesterone treatment was not examined, and accordingly the possibility that the small amount of *allo*pregnane-3α:20α-diol isolated had some endogenous source could not be excluded.

Isolation of pregnane-3α:20α-diol and allopregnane-3α:20α-diol from the urine of pregnant women

The result of the above-mentioned experiment indicated that the application of similar methods to the urine of pregnant women might yield reliable information concerning the relative amounts of pregnane-3α:20α-diol and *allo*pregnane-3α:20α-diol present.

The neutral ether-soluble fraction from 98 l. of pooled acid-hydrolysed late pregnancy urine was prepared in the usual manner. This fraction (10·5 g.) was dissolved in 500 ml. benzene and poured through a column containing 300 g. Al_2O_3. After exhaustive elution with benzene and with ether the desired fraction was eluted with acetone. The residue obtained on evaporation of the acetone was deeply pigmented and somewhat oily; accordingly it was decided to purify it further by the precipitation procedure of Astwood & Jones (1941) as modified by Sommerville, Marrian & Kellar (1948) before proceeding to the benzene extraction.

The diol fraction was dissolved in 1000 ml. ethanol and 4000 ml. water was added slowly while maintaining the temperature of the mixture at 75°. The mixture was then allowed to stand in the incubator at 37° overnight. After the addition of 6 g. Supercel filter aid the mixture was shaken vigorously, and 2 hr. later filtered with suction through a pad of Supercel on a sintered-glass funnel. The diol fraction obtained by the elution of the Supercel pad with boiling ethanol and evaporation of the latter was still highly pigmented; accordingly it was treated with Norit charcoal in boiling ethanolic solution. The ethanolic filtrate on evaporation to dryness yielded 2·55 g. of a brown crystalline solid.

This material was refluxed for 2 hr. with 200 ml. benzene, and after standing at room temperature overnight the solid was filtered off. The latter was returned to the original flask while the benzene filtrate was poured through a column containing 1 g. Al_2O_3. The desired fraction was obtained by elution with 5% (v/v) methanol in ether after exhaustive elution with benzene and with ether. The material obtained by evaporation of the methanol-ether eluate was dissolved in a small volume of methanol and set aside to crystallize at room temperature over a period of 10 days. The solid crystalline material thus obtained was recrystallized from methanol and yielded 21 mg. of thick crystalline rods melting at 235–242°. This material did not significantly depress the melting point of authentic *allo*pregnane-3α:20α-diol and was evidently a somewhat impure specimen of the latter.

By further treatments of the benzene-insoluble fraction in the same manner with benzene and by reworking the methanol mother liquors, additional crops of thick crystalline rods melting at 237–242° (not depressing the m.p. of authentic *allo*pregnane-3α:20α-diol) and amounting to 31·5 mg., were obtained.

The final benzene-insoluble fraction was purified by the modified Astwood-Jones precipitation method as previously described, using 250 ml. ethanol and 1000 ml. water. The precipitated material after a further leaching with benzene weighed 1·70 g., melted at 235–237° and on admixture with authentic pregnane-3α:20α-diol did not depress the melting point of the latter.

Altogether the 98 l. of urine yielded about 1700 mg. of pregnane-3α:20α-diol and 52·5 mg. of *allo*pregnane-3α:20α-diol, and accordingly the ratio of the amounts of these two steroids isolated was about 30:1. Since the isolation of neither steroid was strictly quantitative, this ratio may not represent the exact relative proportion of the two substances actually present in the urine. Nevertheless, it is improbable that it is grossly in error.

It will be seen that this approximate ratio differs strikingly from that of 2:1 given by Marker *et al.* (1938). Quite apart from this discrepancy, however, consideration of the solubility figures for the two diols presented in the present paper seems to make it quite clear that the latter ratio must be incorrect. If the ratio of the two diols in a crude urinary diol fraction were indeed 2:1, then the solubility figures indicate that the first crop of crystals obtained by crystallization from methanol or ethanol should

consist largely of *allo*pregnane-3α:20α-diol: common experience, however, is that the first crop of crystals is invariably pregnane-3α:20α-diol.

Some observations on the conjugated form of allopregnane-3α:20α-diol in human pregnancy urine

The ready isolation of *allo*pregnane-3α:20α-diol, uncontaminated by pregnane-3α:20α-diol, from the hydrolysis products of the 95% aqueous acetone filtrate obtained in the preparation of 'sodium pregnanediol glucuronidate', clearly showed that the ratio of pregnane-3α:20α-diol to *allo*pregnane-3α:20α-diol must be considerably lower than 30:1 in this fraction. The relative enrichment of this fraction in *allo*pregnane-3α:20α-diol suggested either that the latter is present in urine in the free state or that its conjugated form is less completely precipitated from aqueous solution by acetone than is 'sodium pregnanediol glucuronidate'.

To test the first alternative the neutral ether-soluble fraction from 30 l. of fresh unhydrolysed human pregnancy urine was examined for the presence of *allo*pregnane-3α:20α-diol. None was detected, and it is accordingly concluded that the substance is not present in urine in the free unconjugated state.

To test the second alternative two batches of 'sodium pregnanediol glucuronidate' were hydrolysed with acid and the neutral acid-soluble hydrolysis products examined for *allo*pregnane-3α:20α-diol. The first batch, which had been purified by two acetone purifications, yielded significant amounts of *allo*pregnane-3α:20α-diol. A second batch, made up from various samples of 'sodium pregnanediol glucuronidate', each of which had been purified by at least two acetone precipitations, was subjected to three further acetone precipitations. The hydrolysis products of the material finally precipitated yielded no *allo*pregnane-3α:20α-diol.

This experiment is admittedly inconclusive since the two batches of 'sodium pregnanediol glucuronidate' which were examined had different origins. However, the results obtained, considered together with the initial isolation of *allo*pregnane-3α:20α-diol from the 95% acetone-soluble fraction, suggest that the conjugated form of *allo*pregnane-3α:20α-diol is more soluble in 95% acetone than 'sodium pregnanediol glucuronidate' and can be separated from the latter by repeated precipitation from water with acetone.

SUMMARY

1. A simple method for the isolation of *allo*pregnane-3α:20α-diol from human urine has been devised. Evidence is presented to suggest that about 70% of the *allo*pregnanediol actually present can be isolated by this method.

2. The ratio of pregnane-3α:20α-diol to *allo*pregnane-3α:20α-diol in a mixed sample of the urine of pregnant women has been found to be about 30:1.

3. *allo*Pregnane-3α:20α-diol is not present in the free state in human pregnancy urine. Preliminary evidence suggests that the *allo*pregnane-3α:20α-diol conjugate in crude 'sodium pregnanediol glucuronidate' may be completely removed by repeated precipitation from aqueous solution with 20 vol. of acetone.

The authors are indebted to the Medical Research Council for a grant out of which the expenses of this work were defrayed; to Dr J. W. Minnis for the micro-analyses; to Prof. R. J. Kellar for providing facilities for the collection of urine; and to Mrs T. I. Kyle for much technical assistance.

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Biochemistry of Locusts

6. THE OCCURRENCE OF A FLAVIN IN THE EGGS AND OF A PTERIN IN THE EYES OF THE AFRICAN MIGRATORY LOCUST (*LOCUSTA MIGRATORIA MIGRATORIOIDES* R. & F.) AND THE DESERT LOCUST (*SCHISTOCERCA GREGARIA* FORSK.)

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(*Received* 10 *November* 1950)

Previous reports in this series (Goodwin & Srisukh, 1949, 1950, 1951) have been concerned with the major pigment components of locusts; the present paper is concerned with the identification of two minor yellow components, namely, a flavin, in all probability riboflavin, in the eggs and a pterin in the eyes. They occur in both species examined (*Locusta migratoria migratorioides* R. & F. and *Schistocerca gregaria* Forsk.). Riboflavin has previously been reported in *Schistocerca* eggs by Gourevitch (1937) and Busnel & Drilhon (1942) on the basis of fluorescence tests only. It has also been reported in the eggs of another Acridid, *Melanoplus differentialis*, by Bodine & Fitzgerald (1947). Busnel & Drilhon (1942) have stated that pterins occur in *Schistocerca* eyes.

EXPERIMENTAL

Extraction of the flavin from locust eggs. Newly laid eggs or eggs removed from the body cavity of mature females were crushed in a mortar with acid-washed silver sand and ground with small portions of acetone, containing 1 % (v/v) of conc. HCl, until no further pigment was extracted. The combined extracts were concentrated *in vacuo* to one-third their original bulk, then diluted with an equal volume of water and extracted with light petroleum (b.p. 40–60°) to remove the β-carotene which is present in eggs in considerable amounts (Goodwin, 1949). The small amount of light petroleum remaining in the pale-yellow aqueous phase was removed *in vacuo* and 0·1 N-NaOH added until the solution was just acid to litmus. The solution which was reddish-brown was chromatographed on acid-washed talc using water to develop the chromatogram. Two zones separated, the upper being greyish and the lower larger zone being yellow; the upper zone was discarded and the yellow pigment eluted with absolute ethanol. This solution was then examined for riboflavin.

Extraction of the pterin from locust eyes. The compound eyes, which were removed from the head simply by making an incision round the eyes and lifting them out with small forceps, were crushed with acid-washed silver sand and extracted a number of times with small portions of water, about 20 ml. being required to extract completely sixty pairs of eyes. The resulting light purplish extract was filtered through a G 4 sintered-glass crucible and yielded, on standing, a lemon-yellow slightly turbid solution. This solution was then shaken twice with an equal volume of light petroleum, to remove carotenoids, and then evaporated to dryness *in vacuo*. The dry solid was extracted with ethanol to remove final traces of lipid material and then dissolved in water to yield a clear lemon-yellow solution. The main pigment was then separated from a small amount of yellow impurity by chromatography on acid-washed talc using water as the developer. The minor yellow component separated from the main pigment and passed rapidly into the filtrate. The pigment was eluted with water, the water removed *in vacuo*, and the properties of the residue recorded in as much detail as possible considering the very minute amounts of material available. A number of batches of eyes of both species was examined in this way, with identical results in every case.

RESULTS AND DISCUSSION

The flavin in locust eggs

The yellow water-soluble pigment obtained from locust eggs was shown to be indistinguishable from riboflavin in a number of ways. Its absorption spectrum (Fig. 1) over the range 550–350 mμ. was

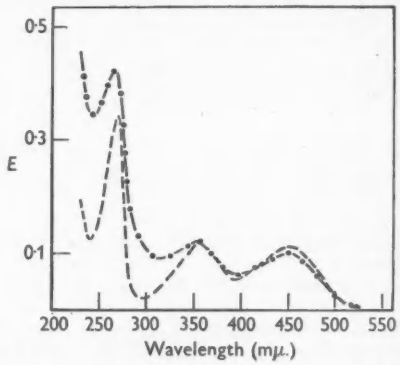

Fig. 1. A comparison of the spectrum of the pigment extracted from locust eggs with that of an authentic specimen of riboflavin. –·–·–·–, Locust pigment; – – – –, riboflavin.

indistinguishable from that of an authentic specimen of riboflavin (supplied by British Drug Houses Ltd.). At wavelengths below 350 mμ. slight deviations were observed, which were consistent with the

presence of small amounts of absorbing impurities in our pigment; the position of the short-wave band was, however, still identical with that of riboflavin (268 mμ. in 0·1 N-NaOH). The pigment was also subjected to the following tests: when dissolved in water or ethanol the pigment exhibits a bright yellow fluorescence in ultraviolet light, whilst in alkaline solution the fluorescence disappears as it does on the addition of sodium dithionite ($Na_2S_2O_4$), to the aqueous or ethanolic solutions. In the latter case the visible yellow colour also disappears, but on shaking in air both the original yellow colour and fluorescence return. Further, the original fluorescence was not destroyed when an aqueous solution of the pigment was treated with acid potassium permanganate and the excess potassium permanganate destroyed with hydrogen peroxide. All these tests are characteristic of riboflavin.

This demonstration that the flavin present in the newly laid locust eggs of both species is in all probability riboflavin, is interesting, for other investigations are not consistent regarding its occurrence in the newly laid eggs of other insect species. Bodine & Fitzgerald (1947) report that it is present in the newly laid eggs of *Melanoplus differentialis*, but that it disappears during incubation, probably owing to its conversion into pterins. Polonovsky & Busnel (1949), on the other hand, consider the opposite state of affairs obtains in the eggs of *Bombyx mori*; these eggs when newly laid apparently contain pterins, which are converted into riboflavin during embryonic development.

The pterin from the eyes of locusts

The pigment, purified as described above, was easily soluble in water, mineral acids, glacial acetic acid, alkalis and ethanol acidified with hydrochloric acid; it was only very sparingly soluble in absolute ethanol and was insoluble in chloroform and ethyl ether. It had a faintly acid reaction, gave a negative murexide test, gave with ammoniacal silver nitrate a brownish precipitate soluble in dilute nitric acid, and, after boiling in faintly acid solution with a 10 % (w/v) copper sulphate solution, gave on standing a yellow precipitate. The original colour was not altered on treatment with zinc and hydrochloric acid. The pigment exhibits an absorption spectrum with the main maximum located at slightly different wavelengths according to the pH of the solvent namely: 288 mμ. in phosphate buffer of pH 7, 292 mμ. in 0·1 N-sodium hydroxide, and 278–280 mμ. in glacial acetic acid; a very wide inflexion occurs at longer wavelengths beginning at about 330 mμ., but only in the phosphate buffer is a slight band ($\lambda_{max.}$ about 345 mμ.) observed. The full curves are recorded in Fig. 2. In ultraviolet light the pigment fluoresces bluish-green when dissolved in distilled water (pH 6·6) or 0·1 N-sodium hydroxide and blue to greenish-blue in glacial acetic acid and dilute mineral acids. The fluorescence is not destroyed by treatment with sodium dithionite, acid potassium permanganate, exposure to ultraviolet light, or heating on a water bath with 10 % (v/v) sulphuric acid for 5 min.; it was suppressed at high pH values (N-sodium hydroxide and higher concentrations). These reactions are characteristic of pterins (Becker & Schöpf, 1936).

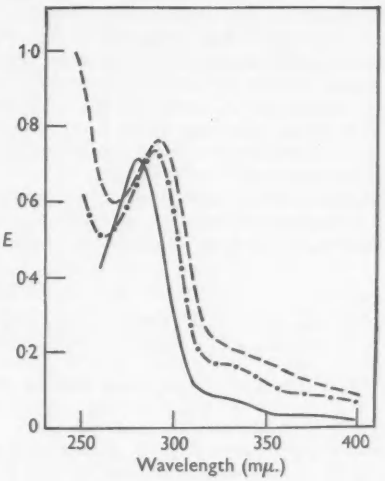

Fig. 2. The absorption spectrum of the pterin-like pigment extracted from locust eyes. –·–·–, In phosphate buffer pH 7; – – – –, in 0·1 N-NaOH; ——, in glacial acetic acid.

If an aqueous solution of the pigment was chromatographed on a column of alumina (Spence, Grade 'O'), through which water had previously been run, the pigment was adsorbed as an orange-brown zone at the top of the column which could be eluted with 0·1 N-sodium hydroxide, glacial acetic acid or 0·5 N-ammonium hydroxide; during development of the column with ammonia solution the orange-brown zone fluoresced bright yellow. The pigment was adsorbed as an orange-yellow band showing bright yellow fluorescence when a solution in methanol containing 0·1 % (v/v) of conc. hydrochloric acid was chromatographed on a column of alumina previously treated with the solvent. These chromatographic observations also indicate the presence of a pterin (Becker & Schöpf, 1936).

The above evidence indicates that the pigment under investigation was, in all probability, a pterin, but owing to the very small amounts of material available, complete identification was not possible. All that can be said is that the properties which have been observed indicate that the pigment more closely resembles xanthopterin than any other re-

ported pterin. In fact, in only two cases is there any divergence in properties, the absorption spectra and the murexide test. Our pigment exhibits a short-wave band with maxima varying from 278 to 292 mμ., according to the pH of the solvent, whilst the consensus of recorded data indicates that the xanthopterin band occurs within the range 255–270 mμ. (see Jacobson & Simpson, 1946); however, Schöpf, Becker & Reichert (1939) have reported the maximum to lie between 275 and 284 mμ. Further, xanthopterin exhibits a band at 375–380 mμ. with an intensity 0·27 of that of the 255–270 mμ. band, whilst our pigment shows only a very wide plateau in this region with about the same relative intensity. A very small band at about 345 mμ. is, however, apparent when phosphate buffer pH 7 is used as solvent. In the murexide test, xanthopterin gives a positive reaction whilst ours did not.

The presence of a fluorescent pigment, not riboflavin but considered to be a pterin, has previously been reported in the eyes of *Schistocerca* by Busnel & Drilhon (1942). On the information available it appears to be different from our pigment, but precise comparison is not possible because Busnel & Drilhon do not describe their methods of extraction and isolation. Pterins have, however, been reported in the eyes of a number of animals including crustaceans, fish, amphibians and reptiles (see Pirie & Simpson, 1946) but not, as yet, in any other insect species.

SUMMARY

1. The presence of a flavin, almost certainly riboflavin, has been observed in the newly laid eggs of the African migratory locust (*Locusta migratoria migratorioides* R. & F.) and of the desert locust (*Schistocerca gregaria* Forsk.).

2. The yellow fluorescent pigment occurring in the eyes of both species has been identified as a pterin; it closely resembles xanthopterin.

We wish to thank the Anti-Locust Research Centre for supplies of locusts and a grant towards laboratory expenses.

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Biochemistry of Locusts

7. A NOTE ON THE EFFECT OF BREEDING TEMPERATURE ON THE CAROTENOID CONTENT OF LOCUSTS (THE AFRICAN MIGRATORY LOCUST *LOCUSTA MIGRATORIA MIGRATORIOIDES* R. & F. AND THE DESERT LOCUST, *SCHISTOCERCA GREGARIA* FORSK.)

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Variations in the temperature at which locusts are reared have a profound effect on their appearance, low temperatures (28°) producing very dark insects, and high temperatures (40°) very pale insects (Parker, 1928; Husain & Mathur, 1936; Jones, 1950). The Anti-Locust Research Centre kindly placed at our disposal a number of specimens of *Locusta migratoria migratorioides* R. & F. and *Schistocerca gregaria* Forsk., reared at different temperatures, and these have been examined quantitatively for insectorubin and for the carotenoids, β-carotene and astaxanthin. The results obtained for insectorubin were incorporated into the general report on the distribution of this pigment in solitary and gregarious locusts (Goodwin, 1950). The corresponding report on carotenoid distribution had, however, already been published (Goodwin, 1949) when these insects became available; consequently, for the sake of completeness, the results on the carotenoid distribution in locusts bred at different temperatures are now reported in this note.

Table 1. *Carotenoid distribution in gregarious* Schistocerca *reared at different temperatures*

Breeding temperature (°)	No. of insects examined	β-carotene $E^{1\%}_{1\,cm.}$	β-carotene Amount per insect (μg.)	Astaxanthin $E^{1\%}_{1\,cm.}$	Astaxanthin Amount per insect (μg.)
			5th instar		
28	4	0·028	17	0·013	10
35	6	0·029	11	0·024	9
40	8	0·081	25	0·025	7
			Immature adults		
28	20	0·031	17	0·012	8
35	12	0·033	18	0·015	10
40	—	—	—	—	—
			Mature adults		
28	—	—	—	—	—
35	8	0·065	50	0·009	10
40	4	0·072	29	0·024	12

EXPERIMENTAL

The locusts were reared in a thermostatically controlled room in London and sent alive to Liverpool.

The carotenoids were extracted, chromatographically separated and assayed according to the methods previously employed (Goodwin, 1949). During the previous investigation it was found that no differential accumulation of carotenoids occurred in various regions of the body; consequently, in this study the complete gutted bodies were used and separate carotenoid determinations on head and pronotum, body, legs and wings were not carried out.

RESULTS AND DISCUSSION

The results obtained for the β-carotene and astaxanthin content of 5th instar hoppers and immature adults of *Schistocerca* reared at different temperatures are recorded in Table 1. The values reported in Table 1 for insects reared at the normal temperature (35°) are mainly based on those previously recorded (Goodwin, 1949), although a number of more recent determinations have also been included.

The most important conclusion which can be drawn from a study of Table 1 is that variation in breeding temperatures has no appreciable effect on the amounts of β-carotene and astaxanthin which *Schistocerca* accumulate. Similar experiments on a more restricted scale using *Locusta* gave identical results, as did experiments with *Schistocerca* maintained at 40° during the day and 28° during the night. This constancy of pigment production is maintained in spite of the fact that, especially in the case of hoppers, the weights of the insects vary considerably with breeding temperature.

This failure of the locust carotenoids to respond to environmental temperature is in contrast to the very marked response observed with the two other major pigments, melanin and insectorubin (Goodwin, 1950). In hoppers, a low breeding temperature (28°) considerably stimulates insectorubin and melanin production, the former by as much as 400%; in adults, from which melanin is absent, insectorubin production is stimulated to the same extent. A breeding temperature of 40°, on the other hand, almost completely inhibits melanin and insectorubin synthesis. The present observations also confirm the previous conclusion (Goodwin & Srisukh, 1950) that, apart from mature males and 5th instar *Schistocerca*, carotenoids play little, if any, part in locust coloration.

SUMMARY

Variations between 28° and 40° in the breeding temperature of locusts (*Schistocerca* and *Locusta*) have no effect on the astaxanthin and β-carotene content of the insects.

Thanks are due to the Anti-Locust Research Centre for supplying the locusts and for a grant towards laboratory expenses.

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Purification of Horse-Radish Peroxidase and Comparison of its Properties with those of Catalase and Methaemoglobin

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Although the peroxidatic properties of animal and vegetable extracts were recognized already by Schönbein (1863) and ascribed by Bourquelot (1898) to special indirect oxidizing enzymes which Linossier (1898) named peroxidases, the first successful isolation and purification of an enzyme in this category was achieved between 1917 and 1924 by Willstätter and his co-workers. They described the main properties of horse-radish peroxidase and greatly improved the method of estimation of enzyme activity, expressed in terms of 'Purpurogallinzahl', P.Z. or purpurogallin number, P.N. (for a definition see p. 89). The unusually high values of P.N. recorded by Willstätter & Pollinger (1923) can only be ascribed to some error in their calculations. Thus if the total iron (0·064 %) found in their preparation with P.N. = 3070 belongs to the prosthetic group of the enzyme, then, in the light of our present knowledge, it can be estimated that the purity of their most active preparation was probably 40–50 % and that the P.N. values given in their paper are too large by a factor of about 5. However, to obtain at that time a 40 % pure enzyme was in itself a remarkable achievement. It not only paved the way for further study of peroxidase, but stimulated similar work on other enzymes at a time when it was still believed (Warburg, 1928) that owing to their inherent fragility all attempts to isolate and purify intracellular enzymes would have no chance of success. Willstätter's classical study of peroxidase was followed by extensive work on the purification of this enzyme with a view to determining its chemical nature.

The presence of haematin in horse-radish root itself (Keilin, 1927) and in enzyme preparations of widely different purities (Kuhn, Hand & Florkin, 1931; Elliott & Keilin, 1934; Sumner & Howell, 1936) suggested the possibility that the enzyme is a haematin compound. However, it was only after further knowledge had been gained of the properties of methaemoglobin and especially of the compounds it forms with hydrogen sulphide (Keilin, 1933), with sodium azide (Keilin, 1936) and above all with peroxides (Keilin & Hartree, 1935) that the haematin nature of peroxidase was definitely established by Keilin & Mann (1937). This opened the way for a much more precise study of the properties of the enzyme and of the kinetics of its reactions.

Peroxidase offers one of the most instructive examples for the study of the chemical structure of an enzyme. Thus the criteria upon which the haematin-protein nature of peroxidase as well as its close relationship to catalase and to methaemoglobin were established (Keilin & Mann, 1937) can be summarized as follows: (1) Presence in all peroxidase preparations, from horse-radish root itself to the most highly purified enzyme, of a pigment with an acid methaemoglobin-like absorption spectrum. (2) Proportionality between the intensities of the absorption spectra of this methaemoglobin-like pigment or of its pyridine-haemochromogen derivative and the activities (P.N.) of different enzyme preparations. (3) Formation of spectroscopically well defined compounds between this pigment and inhibitors of peroxidatic activity such as potassium cyanide, hydrogen sulphide, sodium fluoride and nitric oxide. (4) Formation of well defined compounds between this haematin pigment and hydrogen peroxide which is the substrate of the enzyme. (5) Rapid decomposition of haematin pigment-peroxide complex and the liberation of the pigment on addition of 'acceptors' like pyrogallol, quinol, ascorbic acid and others which can undergo peroxidatic oxidation. (6) Demonstration of the above reactions between the enzyme and inhibitors or substrate (i.e. hydrogen peroxide) not only with purified enzyme but also with crude root extracts and even slices of horse-radish root.

The peroxidase-peroxide compound provided the first example of direct visual demonstration of union between an enzyme and its substrate. According to the amount of hydrogen peroxide added two distinct red compounds are formed with somewhat different absorption spectra. On the other hand, there is no spectroscopically detectable reaction between peroxidase and substances such as pyrogallol, quinol, etc. Peroxidase also resembles methaemoglobin in changing into a redder alkaline form at higher pH (>10) and in being reduced by sodium dithionite ($Na_2S_2O_4$) to a ferrous form which can combine with carbon monoxide.

The purest preparation of Keilin & Mann (1937) had P.N. about 950 and contained 1·2 % haemin. It was also shown that milk peroxidase is also a methaemoglobin-like compound which will combine with potassium cyanide, sodium fluoride and hydrogen

peroxide and, after reduction ($Na_2S_2O_4$), with carbon monoxide.

A large-scale preparation of horse-radish peroxidase described by Theorell (1942*a*) enabled him to isolate the enzyme as a pure crystalline material containing 13·2% nitrogen and 1·48% haemin, estimated as pyridine haemochromogen. The molecular weight of the enzyme calculated from the sedimentation and diffusion constants was found to be 44,100 (Theorell, 1942*b*) while the catalytic activity or P.N., estimated in the usual way, was about 930, the highest value reached being 1100. With this preparation Theorell corroborated the main results of the previous work (Keilin & Mann, 1937), especially the haematin nature of peroxidase and the compounds it forms both with its inhibitors and with peroxide. However, in addition to the two red peroxidase-peroxide compounds previously described, Theorell discovered a third enzyme-substrate compound which is of greenish colour and shows an absorption band at about 652 mμ. (Theorell, 1941). Since this green compound is the first enzyme-substrate complex formed, the numbering of these derivatives has been modified accordingly. Thus the first green, the first and second red compounds are now referred to as peroxidase-peroxide compounds I, II and III respectively. Theorell (1940) was also able to split peroxidase into its protein and haematin components and to reconstruct active enzymes from the native protein and different haematins (Theorell, Bergström & Åkeson, 1943).

In the present paper a description will be given of a comparatively simple method of preparation of pure horse-radish peroxidase. The spectrophotometric curves of peroxidase and its known reversible derivatives will be given both for the visible and the Soret region, and the properties of peroxidase will be compared with those of catalase and methaemoglobin.

Nomenclature. The catalytic system in which peroxidase is involved is composed of (1) enzyme: peroxidase, (2) substrate: hydrogen peroxide, and (3) a substance undergoing peroxidatic oxidation, usually called an 'acceptor', but which will be referred to in this paper as a hydrogen donor.

EXPERIMENTAL

Methods

Activity of peroxidase preparations was determined through the purpurogallin test as carried out by Elliott & Keilin (1934), Keilin & Mann (1937) and others, which is a slight modification of the original method devised by Willstätter & Stoll (1918). Suitably diluted enzyme (0·1–0·5 ml.) was added to 500 ml. glass-distilled water at 20° containing 1·25 g. resublimed pyrogallol. No commercially available samples of pyrogallol were satisfactory unless resublimed under reduced pressure (approx. 40 mm. Hg). H_2O_2 (0·4 ml. 6% w/v) was added with stirring, and after exactly 5 min. the reaction was stopped by the addition of 5 ml. 5 N-H_2SO_4. The yellow purpurogallin was extracted by shaking with three lots of ether (80, 30, 30 ml.) which had been freed from peroxide by distillation over $FeSO_4$ + CaO. The volume of the ether solution was measured, the solution dried with anhydrous Na_2SO_4 and the concentration of purpurogallin estimated in the Hilger Spekker absorptiometer using 1 cm. cells and Ilford 602 filters. The instrument was calibrated with ethereal solutions of twice recrystallized purpurogallin. The quantity of enzyme taken for the test should be such that less than 10 mg. purpurogallin is produced.

Enzyme activity is expressed in terms of purpurogallin number, P.N. $= w/vd$, where w = mg. purpurogallin formed under the above conditions from v ml. of enzyme solution and d = the dry weight in mg. of 1 ml. of solution.

The number of enzyme units in a peroxidase preparation, E.U. $= Vw/1000v$, where V is the total volume of the peroxidase solution.

Dry weight. Solution (1–2 ml.) was evaporated to constant weight at 105° in a weighing bottle. Prolonged heating was sometimes necessary and the dry residues were often hygroscopic.

Nitrogen was estimated by the micro-Kjeldahl method, the NH_3 being distilled in the Markham (1942) apparatus into HCl which was back titrated with 0·01 N-$Ba(OH)_2$.

Haemin concentration was estimated as follows: A suitable volume (1–2 ml.) of the preparation was pipetted into a 10 ml. volumetric flask containing 0·4 ml. N-NaOH to which 2 ml. pyridine was then added followed by about 50 mg. $Na_2S_2O_4$. The flask was made up to the mark with distilled water, stoppered, well mixed and allowed to stand 0·5 hr. The concentration of haemin, as pyridine haemochromogen, was measured in the Spekker absorptiometer using 1 cm. cells and Ilford 605 filters. The blank cells contained all ingredients except peroxidase. For calibrating the intrument haemin was recrystallized from pyridine-$CHCl_3$-acetic acid. A stock 0·1% solution of this haemin was made up in 0·1 N-NaOH and measured volumes of it were taken for preparation of standard haemochromogens. The final concentration of haemin must not exceed 4 mg./100 ml. The estimation of haemochromogen by spectrophotometric measurement of the height of the sharp α-band at 556 mμ. was considered to be insufficiently reliable since a slight shift of the band can introduce a large error. An additional error may arise from variation in the height of the α-band according to whether protein is present or not. The measurement with a Spekker absorptiometer as described above obviates these difficulties.

Throughout this paper results are expressed as '% haemin', i.e. ferric protoporphyrin chloride, since this compound is weighed out to make the standards. In earlier papers results have been given as '% haematin' (ferric porphyrin hydroxide, formed on dissolving haemin in alkali), although in fact the analyses were based on haemin. Since in peroxidase one of the two residual valencies of Fe is linked to the protein and the other either to hydroxyl (Theorell & Paul, 1944) or to a molecule of water (Keilin & Hartree, 1949), the molecular weights of haemin and of the prosthetic group of peroxidase are 652 and 635–636 respectively. Hence haemin values will be approx. 3% higher than the true values for the accepted prosthetic group. However, for the sake of consistency we shall refer to 'haemin content'.

Spectroscopic observations were carried out with the Zeiss microspectroscope and Hartridge reversion spectroscope.

Absorption curves of peroxidase and its derivatives were determined with the Beckman photoelectric spectrophotometer using 1 cm. cells. The following units are used in plotting the curves: Optical density $D=\log_{10}(I_0/I)$; extinction coefficient $K=D/l$; molar extinction coefficient $\epsilon=D/lc$, where l = optical depth in cm. and c = molar concentration of haemin. In an earlier paper (Keilin & Hartree, 1945*a*) we plotted the absorption constant $\beta=\log_m(I_0/I)/lc$, where c is expressed as g.mol./ml. The absorption coefficient is therefore 2303 times greater in terms of β than in terms of ϵ.

Estimation of peroxidase in horse-radish roots and crude enzyme extracts was carried out by means of a microspectroscope ocular and double wedge trough as described by Elliott & Keilin (1934) and illustrated by Keilin & Wang (1946). The principle of the method consists in matching the absorption bands of pyridine haemochromogen obtained from peroxidase against pyridine haemochromogen prepared from crystalline haemin, or in matching the absorption bands of one of the reversible derivatives (i.e. fluoride) of peroxidase in slices or extracts with those of the corresponding derivative obtained from pure peroxidase.

Calcium phosphate gel. To a 10 l. glass jar containing 1 l. tap water and 250 ml. 0·6 M-$CaCl_2$ was added, with stirring, 250 ml. 0·4 M-Na_3PO_4 followed by sufficient N-acetic acid to bring the pH to about 7·3. The jar was filled with water and the precipitate washed six times by decantation. It was finally collected in centrifuge tubes and suspended in tap water to give about 400 ml. of a thick cream.

Catalase was prepared from horse liver as previously described (Keilin & Hartree, 1945*a*).

Notatin was the same material as described in a previous publication (Keilin & Hartree, 1945*b*).

Alternative procedures. For the estimation of purpurogallin concentration the Spekker absorptiometer can be replaced by the King photoelectric colorimeter (with blue filter) or by any standard visual colorimeter, while the King instrument with the green filter gives very reliable results in the estimation of haematin by the above method. Since it is in the visible rather than in the Soret region of the spectrum that the reactions of peroxidase can be followed, a visual spectrophotometer (e.g. Hilger-Nutting) is adequate for quantitative studies of peroxidase.

Isolation of peroxidase from horse-radish root

Preparation of crude extract. Roots of wild horse-radish (*Cochlearia armoracia* L.) were gathered in spring at the time when the first shoots are appearing and the interference by root polysaccharides during fractionation is at a minimum. Well washed roots, after removal of damaged portions, were cut into 2–4 cm. long sections and passed through a mechanical mincer. The juice was extracted from 50 kg. mince with a stainless steel hydraulic press and water, equal in volume to the press-juice, was well mixed with the mince which was then pressed out again. The process was repeated once more and the three extracts were mixed. When dealing with large quantities of root it is advisable to work in the open air, or alternatively to use a respirator for protection against the strongly lachrymatory mustard oil (allyl *iso*-thiocyanate) which, during the mincing, is rapidly liberated from the glucoside sinigrin by a specific glucosidase.

First fractionation. About 69 l. of the above crude extract was treated with 47 kg. $(NH_4)_2SO_4$ and the mixture (0·92 saturation) left standing overnight. Next day the precipitate which had risen to the top was collected, placed on two 27 cm. Büchner funnels containing a bed of kieselguhr and the residual fluid was sucked off. The hard cakes were inverted on to sheets of paper, the kieselguhr scraped off and the precipitate suspended in 2 l. water. Since a suspension can only be obtained by kneading with the fingers it is advisable, on account of the residual mustard oil, to wear rubber gloves and to work in a well ventilated fume cupboard. The suspension was placed in cellophan tubing and dialysed for 3 days against running tap water to remove $(NH_4)_2SO_4$. The contents of the tubing were then filtered to give 4 l. of solution *A*. This solution was treated with 1248 g. $(NH_4)_2SO_4$ (0·5 saturation) and the precipitate (*b*) which was formed only after several hours was filtered off. The filtrate (4600 ml.) was brought to 0·85 saturation by addition of 1150 g. $(NH_4)_2SO_4$ when a sticky precipitate (*c*) appeared gradually and adhered to the glass container. The two precipitates *b* and *c* were separately dissolved in water and dialysed until salt-free, giving respectively the solutions *B* and *C*. Solution *B* contained much coloured impurity, which gave it a greyish-green colour, and had a P.N. of 24. The bulk of the enzyme was in the brown solution *C* which had a P.N. of 129 and contained 2040 E.U. At this stage the fractionation was interrupted while a second batch of horse-radish root (48 kg.) was worked up in the same way to give the corresponding solutions B_1 (P.N. = 32) and C_1 (P.N. = 112; 2100 E.U.).

Further purification of fractions B and B_1. These fractions, which contained 15% of the total enzyme units, were combined to give 1025 ml. solution, treated with 100 ml. tricalcium phosphate gel and centrifuged after 5 min. The supernatant fluid was treated with a further 100 ml. calcium phosphate gel and the gel cakes were discarded. The solution (1050 ml.) was brought to 0·40 saturation by addition of 264 g. $(NH_4)_2SO_4$ after which the precipitate was centrifuged off and discarded since it contained only 89 E.U. of P.N. = 27. A second precipitate obtained by addition of 232 g. $(NH_4)_2SO_4$ (0·70 saturation) was collected on a filter, dissolved in the minimum amount of water and dialysed until completely free of salts. The solution B_2 thus obtained had P.N. = 163 and E.U. = 568.

Isolation of the pure enzyme

The solutions *C*, C_1 and B_2 were now combined to give 2 l. solution *D* (P.N. = 125; E.U. = 4630), the starting material for the next stages of purification.

At all subsequent stages, unless otherwise stated, dialysis was carried out first against running tap water and finally against distilled water. On all occasions where ethanol was present, the liquids and centrifuge tubes were cooled in ice, but the use of a refrigerated centrifuge was found to be unnecessary.

(*a*) *Precipitation with ethanol.* One-third of solution *D* (i.e. 667 ml.) was divided equally among four 1 l. centrifuge tubes to each of which was added 1·5 vol. (250 ml.) 90% ethanol. The tubes were centrifuged for 10 min., the sticky solid was discarded, while the supernatant from each tube was poured into another similar centrifuge tube containing 416 ml. 90% ethanol and 10 ml. 0·6 M-$CaCl_2$; the final concentration of ethanol was thus about 72%. The tubes were immediately centrifuged for 15 min. and the supernatant fluid discarded. The precipitates from the four tubes were suspended in a little distilled water and kept at 0° while the remainder of solution *D* was treated in two lots in the same way. The suspensions of precipitates were finally united and

dialysed overnight against 5 l. distilled water at 5° to give 567 ml. solution *E* (P.N. = 586; E.U. = 4000).

(*b*) *Fractionation with calcium phosphate and ethanol.* Solution *E* was added with stirring to a mixture of 510 ml. water and 76·5 ml. calcium phosphate (37·4 mg./ml.). The calcium phosphate was centrifuged off, washed with 100 ml. water and the cake eventually discarded. (Elution of the cake with 2% potassium oxalate gave a dark greyish solution containing only 56 E.U.) The supernatant solution and washings (1147 ml.) were diluted with an equal volume of 90% ethanol followed by 408 ml. ethanolic calcium phosphate (a mixture of equal volumes of 90% ethanol and the calcium phosphate suspension). The mixture was centrifuged for 10 min. and the cake was immediately suspended in water to a final volume of 200 ml. The supernatant fluid was further treated with two successive lots of ethanolic calcium phosphate (200, 100 ml.) and the practically colourless supernatant was discarded.

(*c*) *Fractionation with* $(NH_4)_2SO_4$. The combined brown calcium phosphate suspensions (500 ml.) were mixed with an equal volume of saturated $(NH_4)_2SO_4$, allowed to stand 1 hr. and centrifuged. The cakes were extracted twice more with small quantities of 0·5 saturated $(NH_4)_2SO_4$ and the washings added to the dark-brown supernatant fluid. The total volume of solution at this stage was 1290 ml. By successive additions of solid $(NH_4)_2SO_4$ to 0·60 saturation (84 g.), 0·68 saturation (68 g.) and 0·85 saturation (171 g.) three precipitates were obtained and filtered off. A little peroxidase was held in solution by the ethanol present even at 0·85 saturation with $(NH_4)_2SO_4$, but by keeping this solution in an open dish overnight a fourth precipitate, containing the remaining enzyme, was obtained.

The first precipitate was rejected, the second was dissolved in water and dialysed to give 222 ml. solution *F* (P.N. = 753; 579 E.U.) while the third and fourth were combined, dissolved and dialysed to give 232 ml. solution *G* (P.N. = 831; 3520 E.U.).

(*d*) *Heat denaturation of impurities followed by* $(NH_4)_2SO_4$ *fractionation.* Solution *G* was diluted with water to 1334 ml. and treated with 306 g. $(NH_4)_2SO_4$ (0·38 saturation). Quantities of $\nearrow$ 200 ml. were heated rapidly to 62°, held at this temperature for 10 min. and rapidly cooled to room temperature. The combined heat-treated solutions were centrifuged to give a grey cake insoluble in water, and a slightly cloudy fluid which was clarified by filtration through Whatman no. 5 paper. (Although centrifuging did not yield a clear fluid, it accelerated the subsequent filtration.) The clear fluid (1340 ml.) was fractionated by successive addition of $(NH_4)_2SO_4$: 168 g. (0·56 saturation), 75 g. (0·65 saturation) and 44 g. (0·70 saturation). The three precipitates were filtered off, dissolved separately in a little water and dialysed to give solutions containing 700, 2550 and 344 E.U. respectively. The second fraction, *H* (0·65–0·70 saturation), had a total volume of 104 ml. All attempts to increase the P.N. and haemin content of fraction *H* by further $(NH_4)_2SO_4$ fractionation were unsuccessful. In fact, the variations between different fractions were within experimental error and the average analytical figures were: haemin 1·61%, nitrogen 13·2%, P.N. = 1220 ± 50. The behaviour of fraction *H* in the ultracentrifuge showed that it was almost homogeneous (see Addendum to this paper, p. 105).

Absorption curves of peroxidase and its derivatives

The positions of the absorption bands of peroxidase and its derivatives have been recorded by Keilin & Mann (1937). The spectrophotometric curves of free peroxidase and 'paraperoxidase' have been given by Theorell (1941, 1942*a*), whereas curves for the Soret bands of the three peroxidase-peroxide compounds and of the peroxidase-fluoride and peroxidase-cyanide compounds have been recorded by Chance (1949*b*).

For measurements of light absorption of peroxidase and its derivatives in the Beckman spectrophotometer, solutions were prepared as shown in Table 1. They were suitable for density determinations at the red end of the spectrum while at lower wavelengths the peroxidase concentration had to be reduced five to twenty times. In making suitable dilutions the concentrations of the other ingredients were kept constant. The absorption curves of peroxidase and of its derivatives except those with H_2O_2 are grouped in two figures. Fig. 1 represents the absorption curves of free peroxidase and of its compounds with fluoride, cyanide and azide, while Fig. 2 shows the absorption of alkaline peroxidase, of peroxidase reduced with $Na_2S_2O_4$ and of the latter after treatment with CO.

The comparison of these curves with those of the corresponding derivatives of methaemoglobin and

Table 1. *Preparation of solutions for spectrophotometric measurements on peroxidase and various derivatives other than the* H_2O_2 *compounds*

(A stock solution of peroxidase (0·386 mM, P.N. = 1220) was used to prepare the following solutions.)

Peroxidase	Other ingredients	Final haemin concn. (μM)	Spectrum obtained
a 2·0 ml. stock	8 ml. 0·25 M-Phosphate buffer, pH 5·6	77·4	Peroxidase
b 3·0 ml., *a*	0·3 ml. 0·7 M-KCN in M-KH_2PO_4	70·3	CN^--peroxidase
c 3·0 ml., *a*	0·6 ml. 2·5 M-NaF	64·5	F^--peroxidase
d 1·0 ml. stock	4·0 ml. 0·7 M-NaN_3 in 0·25 M-acetate buffer, pH 4·25	77·4	N_3^--peroxidase
e 0·6 ml. stock	3·0 ml. 0·6 M-K_2CO_3 (pH 11·4)	64·5	Alkaline peroxidase
f 0·5 ml. stock	4·0 ml. 0·25 M-Phosphate buffer, pH 6·8, 20 mg. $Na_2S_2O_4$*	42·9	Reduced peroxidase
g as *f*	Saturated with CO*	42·9	CO reduced peroxidase

* Solutions covered with layer of paraffin during measurements.

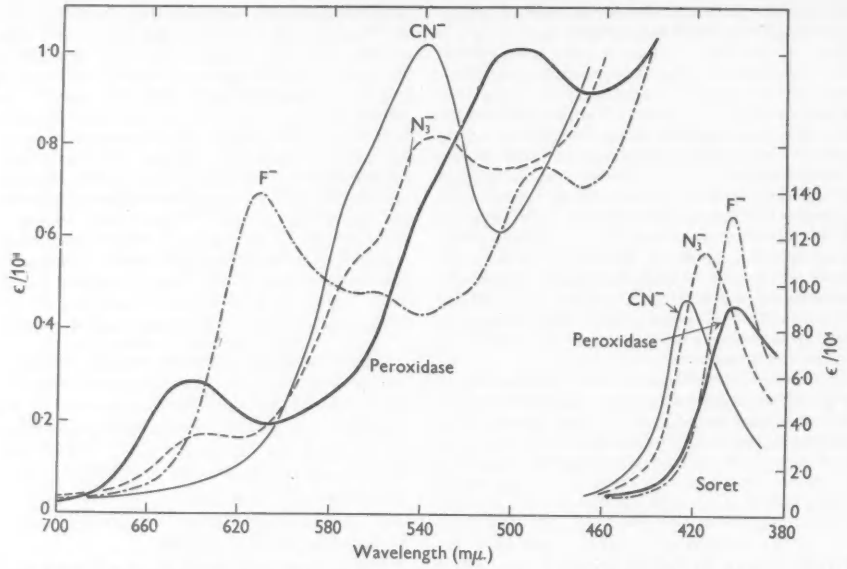

Fig. 1. Absolute absorption spectra of peroxidase and of its fluoride (F^-), cyanide (CN^-) and azide (N_3^-) compounds. For details of solutions used see Table 1. $\epsilon = 1/cl \log_{10} I_0/I$, where c = molar concentration of haematin and l = optical depth in cm. Ordinate scale ($\epsilon/10^4$) reduced 20-fold for Soret bands.

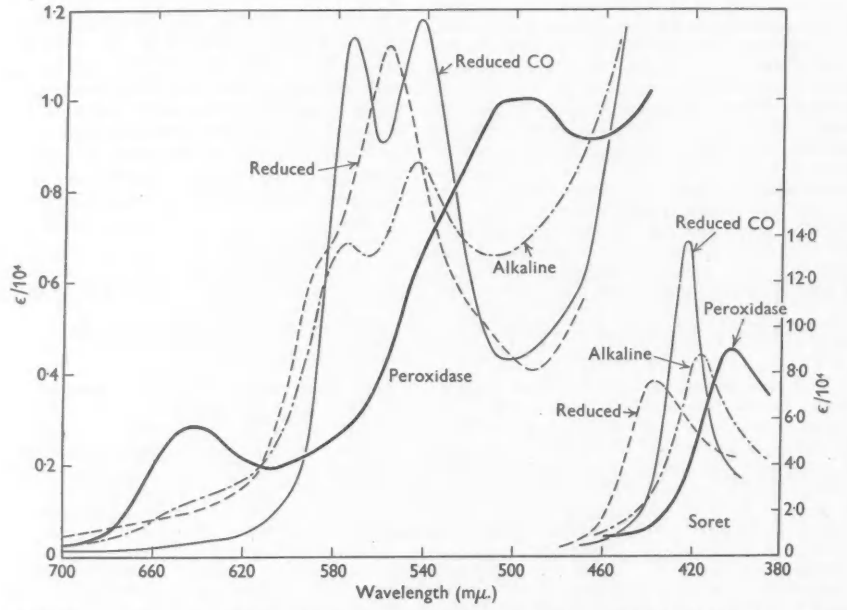

Fig. 2. Absolute absorption spectra of peroxidase, alkaline peroxidase, reduced peroxidase and CO-reduced peroxidase. For details of solution used see Table 1. For definition of units see Fig. 1.

catalase will be discussed at a later stage. Here we shall note only a few characteristic features of the peroxidase curves.

(1) The height of the band in the red of free peroxidase is somewhat greater than that given by Theorell & Paul (1944). Their value for $\beta_{640} = 0{\cdot}585 \times 10^7$ is equivalent to $\epsilon_{640} = 0{\cdot}254 \times 10^4$, whereas our value is $0{\cdot}284 \times 10^4$. A similar proportional increase in the value of ϵ for the Soret band was found on comparing our results (see Fig. 3) with those of Chance (1949*b*).

(2) The azide derivative of peroxidase is only formed below pH 4·5 (Theorell, 1942*c*), while at pH lower than 4 the compound becomes unstable as indicated by a drift in optical density readings.

detection and estimation of peroxidase in roots and in crude extracts is more easily carried out after its transformation to one of these derivatives.

(5) The Soret band of CO-ferroperoxidase is much higher and sharper than the corresponding band in other derivatives of peroxidase.

Peroxidase-peroxide compounds

Peroxidase, as was mentioned previously, forms three compounds with H_2O_2: the green compound I of Theorell (1941) which is a precursor of the two red compounds II and III of Keilin & Mann (1937). Compound II, which shows absorption bands at 555 and 527 mμ., is formed with as little as 1 mol. H_2O_2

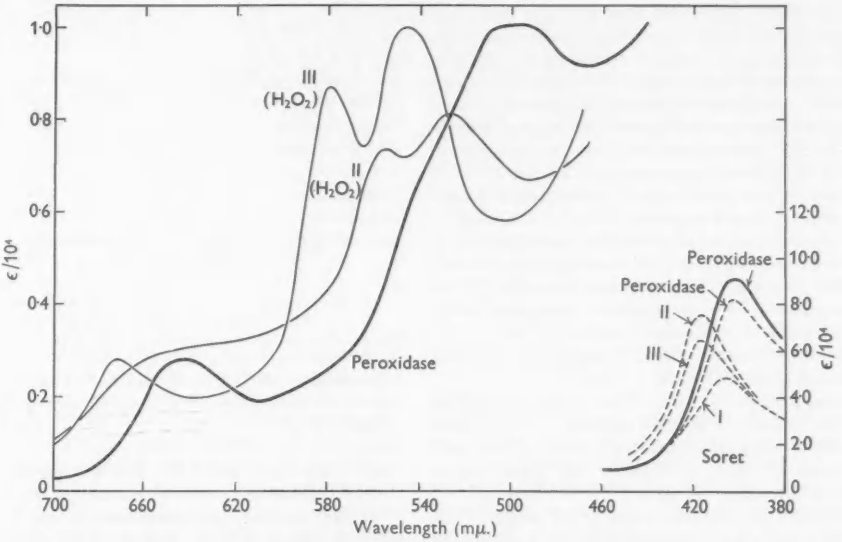

Fig. 3. Absolute absorption spectra of peroxidase and peroxidase-H_2O_2 compounds I, II and III. The pecked lines represent the Soret bands of peroxidase and of its three peroxide complexes as obtained by Chance (1949*b*). The full lines are based on the results of the present investigation.

(3) On addition of fluoride to peroxidase the absorption band in the red is replaced by a much stronger and sharper band at 612 mμ. which is, however, not as strong as that of the cyanide compound or of reduced peroxidase and its CO derivative. Nevertheless, peroxidase in horse-radish roots is more readily detected as the fluoride compound than as any other derivative since the main band is at a wavelength where there is no interference from other pigments and, at the same time, it is more intense than the peroxidase band at 641 mμ. The Soret bands of the F^- and CN^- compounds have also been plotted by Chance (1949*b*, Fig. 6).

(4) Since all derivatives of peroxidase show much sharper absorption bands than the free enzyme, the per Fe atom and even in absence of acceptor it rapidly reverts to free peroxidase. On the other hand, the addition of 15–25 molecules of H_2O_2 leads to the formation of compound III which is also red and shows absorption bands at 583 and 546 mμ. Furthermore, a peroxide concentration high enough to yield 100% of complex III will bring about a fairly rapid destruction of the enzyme with an intermediate formation of a compound showing a distinct band in the red at 670 mμ. This compound also appears when any of the peroxidase-peroxide complexes (I, II or III) are repeatedly reformed by successive additions of small amounts of H_2O_2 to peroxidase liberated from the complex. The compound with an absorption band at 670 mμ. resembles

the complex IV described by Chance (1949*b*) as being formed in the reaction between peroxidase and methyl hydrogen peroxide where, in absence of a derivative corresponding to peroxidase-H_2O_2 III, it follows directly the formation of complexes I and II. It is, however, quite conceivable that the band at 670 mμ. belongs to a peroxidase with a modified prosthetic group (a verdo-type as was suggested by Chance) not necessarily combined with peroxide. This complex therefore is not comparable with I, II and III and the term complex IV is hardly appropriate for it.

Spectrophotometric curves of the peroxidase-peroxide complexes (Fig. 3) were determined by us only for complexes II and III in the visible region. Since the light absorption is constantly changing during these determinations, it is necessary to obtain records of changes of density with time at each of a number of wavelengths and from such records to construct the curves. Under the experimental conditions of these determinations no oxygen bubbles appeared. The Soret bands of all three complexes are taken from a recent paper by Chance (1949*b*).

Peroxidase-peroxide I is the first compound formed when a very small amount of H_2O_2 is added to peroxidase. Direct spectroscopic examination reveals a band at 657 mμ. As the compound is very unstable and rapidly passes into complex II its spectrophotometric curve cannot be recorded by the present method. However, its Soret band, determined by Chance (1949*a*, *b*) using his rapid flow method, is given in Fig. 3.

Peroxidase-peroxide II. The stock peroxidase solution, 4·52 ml., described in Table 1, was diluted with 0·25 M-phosphate buffer, pH 5·6, to 25 ml. and a solution of H_2O_2 (0·00446 M) was made up in 0·125 M-phosphate buffer, pH 5·6. The former solution (2·5 ml.) was mixed with 1 ml. of the latter, the mixture placed immediately in a Beckman absorption cell and readings of density (D) were taken with the minimum delay at 595 and 586 mμ. Records were kept of the times at which each reading was taken, and six further concurrent sets of readings were then taken alternately at the same two wavelengths. Thus during 15 min. seven values of D at definite times after mixing were obtained at each of these wavelengths. The above operations were repeated at seven other series of two wavelengths so that data became available for each of the following sixteen wavelengths: 595, 586, 583, 580, 578, 573, 568, 564, 558, 553, 545, 538, 530, 520, 500 and 470 mμ. In the region above 595 mμ. the changes of D were slower and it was possible to 'scan' the following wavelengths using one peroxidase sample: 750, 700, 680, 672, 660, 640 and 615 mμ. From the various density-time curves, absorption curves at various times were constructed and those for 1·5, 7, 10 and 13·5 min. are given in Fig. 4. The 1·5 min. curve represents a mixture of complexes II and III in which the latter predominates. Since the H_2O_2 slowly decomposes the later curves show the α- and β-bands of II. The ratio of the band heights approached a constant value at 13·5 min. and the corresponding curve may therefore be taken as the nearest approximation to that of peroxidase-peroxide II (see also Fig. 3).

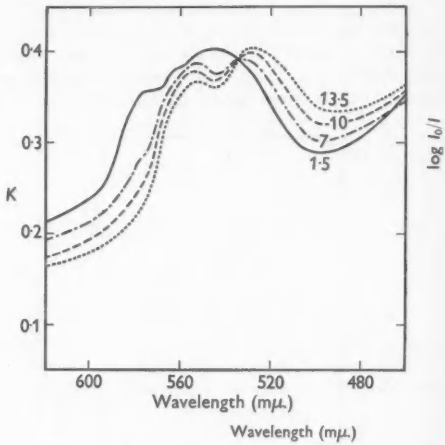

Fig. 4. Changes of absorption spectrum of peroxidase with time (min.) after addition of H_2O_2. Series II experiments. Peroxidase, $5{\cdot}00 \times 10^{-6}$ M; H_2O_2, $1{\cdot}28 \times 10^{-3}$ M; 0·2 M-phosphate buffer, pH 5·6. $K = 1/l \log_{10} I_0/I$, where l = optical depth (1 cm.).

Peroxidase-peroxide III. For the study of this complex the same solutions were used as for complex II except that the H_2O_2 concentration was five times greater. Since D now changes more slowly each peroxidase sample could be used for the study of the density-time changes at four wavelengths instead of two. The region 595–750 mμ., as in the previous experiment, could be covered with one sample of enzyme. It was found that there was a drift in readings during the first minute after which the absorption remained constant until the end of the third minute. Subsequently there was a progressive decrease in D at all wavelengths indicating a destruction of peroxidase. The 3 min. absorption curve, which may be taken as a close approximation to that of complex III, is shown in Fig. 3. Since a slight decomposition of the pigment has occurred the bands as represented are a little too low.

Of the three complexes the peroxidase-peroxide III shows the most distinct absorption spectrum with two bands in the visible region resembling those of methaemoglobin-peroxide. The absorption spectrum of complex II is of similar pattern, but the

bands are less distinct and nearer to the blue. On the other hand, the Soret band of III is lower than that of II. The absorption spectrum of I is of the same pattern as that of free peroxidase, but it has an exceptionally low Soret band (Fig. 3).

A summary of the absorption maxima of different derivatives, based upon the spectrophotometric curves represented in Figs. 1–3, is given in Table 2.

Table 2. *Wavelengths ($m\mu$.) of absorption maxima and (in parentheses) the corresponding values of $\epsilon \times 10^{-4}$ for peroxidase and derivatives*

Peroxidase (Fe^{+++})	641·5 (0·284)	497 (1·003)	403 (9·10)
Alkaline peroxidase	574·5 (0·686)	545 (0·864)	416 (8·85)
Reduced peroxidase (Fe^{++})	556 (1·122)	—	437 (7·73)
Reduced peroxidase CO	572·5 (1·140)	542 (1·177)	423 (13·80)
CN^--peroxidase	538 (1·020)	—	423 (9·39)
F^--peroxidase	612 (0·693)	487·5 (0·755)	404 (13·00)
N_3^--peroxidase	635 (0·170)	534 (0·820)	416 (11·40)

The wavelengths of the absorption bands of the unstable H_2O_2 complexes recorded in Figs. 3, 4 and 6 differ slightly from the figures obtained on direct visual observation with either the microspectroscope or reversion spectroscope (Table 3). This is due partly to the differing experimental conditions and partly to the fact that close agreement between the two methods can only be obtained when the absorption bands are symmetrical.

Effect of low temperature on the absorption spectrum of peroxidase

On freezing and cooling to the temperature of liquid air, the absorption bands of peroxidase and its derivatives are sharpened, greatly intensified and slightly shifted towards the blue end of the spectrum. In this respect peroxidase behaves like some other derivatives of haematin and many other substances showing selective absorption (Keilin & Hartree, 1949, 1950*a*). Freezing in liquid air also makes possible the direct observation in horse-radish root slices of the absorption band in the red of free peroxidase which, owing to its diffuse absorption spectrum, is hardly perceptible at ordinary temperatures.

As already mentioned, peroxidase, like methaemoglobin, shows very marked changes in colour and absorption spectrum with changes in pH. Thus at pH above 11 a reddish alkaline form of peroxidase is formed with bands at 574 and 545 $m\mu$. (Fig. 2). When a neutral solution of peroxidase is cooled its absorption band in the red is intensified and shifted 7–9 $m\mu$. nearer the blue end of the spectrum. On the other hand, when alkaline peroxidase is cooled, its colour and absorption spectrum revert to those of neutral peroxidase with the appearance of the characteristic band in the red. On warming the solution to room temperature the colour and absorption spectrum of alkaline peroxidase rapidly reappear. We observed a precisely similar change on cooling a solution of alkaline methaemoglobin and interpreted it on the assumption that the sixth octahedral position around the iron atom in acid methaemoglobin and also in neutral peroxidase is occupied by a molecule of water. The transition to the alkaline form would then involve not the addition of a hydroxyl ion but rather the dissociation of a proton, as was previously suggested by Coryell, Stitt & Pauling (1937) in the case of methaemoglobin. The shift in equilibrium observed on cooling alkaline peroxidase or alkaline methaemoglobin could thus be ascribed to a suppression of ionization. This is strongly supported by the fact that similar changes in colour can be observed on cooling solutions of some indicators (Keilin & Hartree, 1949). Below a certain temperature cooling affects the colour of indicators by suppressing their ionization, a phenomenon which is general for electrolytes (Harned & Embree, 1934). The spectroscopic study of peroxidase at low temperatures does not support the view put forward by Theorell & Paul (1944) that between pH 5 and 11 the sixth co-ordination place is occupied by a hydroxyl group while at pH 11 two hydroxyl groups are attached to the iron atom by covalent links.

On the so-called 'paraperoxidase'

It was claimed at one time by Theorell (1940, 1942*a*) that horse-radish roots contain two different peroxidase enzymes: peroxidase I or paraperoxidase and peroxidase II or true peroxidase. According to Theorell the characteristic properties of these peroxidases are as follows:

Peroxidase I or paraperoxidase is present in horse-radish roots in variable but always much lower concentrations than those of true peroxidase. It shows an absorption spectrum of the parahaematin type with two bands at 583 and 548 $m\mu$. which on reduction with $Na_2S_2O_4$ are replaced by typical haemochromogen bands lying at 565 and 535 $m\mu$. Peroxidase I is precipitated with picric acid and on cataphoresis at pH 7·5 it moves towards the cathode. Its peroxidatic activity on a haemin basis is of the same magnitude as that of true peroxidase. It catalyses the oxidation of dihydroxymaleic acid directly and this reaction is almost completely inhibited by 10^{-5} M-KCN.

Peroxidase II or true peroxidase shows a methaemoglobin-like spectrum with bands at 640 and

498 mμ.; it is not precipitated by picric acid and on cataphoresis at pH 7·5 it moves towards the anode. For oxidation of dihydroxymaleic acid it requires a carrier such as quinol and this reaction is not affected by cyanide.

These views were extended by Theorell (1942*a*) in his main paper on the subject and in a general review (Theorell, 1943). It was, however, partly withdrawn by Theorell & Åkeson (1942) in a paper dealing with the microdetermination of amino-acids in peroxidase and catalase. Unfortunately this withdrawal was lost in a paper the subject matter of which had no direct bearing on the problem, with the result that the supposed existence in horse-radish roots of two peroxidases has reached books and reviews. Moreover, even in his more recent review, Theorell (1947) considers the so-called paraperoxidase as only 'possibly a derivative of peroxidase' and what is important, in none of his papers or reviews does he refer to his earlier statement that true peroxidase is unable to catalyse directly the aerobic oxidation of dihydroxymaleic acid.

Personal observations. In our experience with many preparations of horse-radish peroxidase we have always obtained only a typical true peroxidase with the characteristic methaemoglobin-like absorption spectrum and even the most purified fractions catalysed the oxidation of dihydroxymaleic acid without the addition of quinol. However, on several occasions we found that when a solution of highly purified true peroxidase was left for a considerable time in an ice chest its methaemoglobin-like absorption spectrum turned gradually into that of a parahaematin. The paraperoxidase thus obtained showed the following properties:

(1) Instead of the normal brown colour the solution was reddish-brown and showed two absorption bands in the visible region lying at about 582 and 543 mμ.

(2) On addition of $Na_2S_2O_4$ a haemochromogen-like spectrum rapidly appeared but with the bands lying nearer to the red end of the spectrum than usual, i.e. at about 565 and 535 mμ. This spectrum was, however, soon replaced by that of typical reduced true peroxidase with a wide, strong band at 556 and a narrow, faint band at 594 mμ.

(3) On addition of KH_2PO_4 and NaF the solution turned greenish and the band of fluoride peroxidase appeared at 612 mμ. while the bands of parahaematin disappeared.

(4) On addition of KCN the solution turned red and showed the characteristic cyan-peroxidase absorption spectrum.

(5) On addition of H_2O_2 the solution showed in turn the absorption spectra of peroxidase-peroxide compounds II and III.

(6) The peroxidatic activity of the solution when the parahaematin spectrum was fully developed was only 38 % lower than that of the original peroxidase solution.

All this clearly shows that paraperoxidase is a transformation product of true peroxidase. What is important, however, is that both compounds react with fluoride, cyanide and a reducer ($Na_2S_2O_4$) to yield similar derivatives. It is therefore conceivable that paraperoxidase can, under certain conditions, revert to the original product and that its formation is due to a partial and reversible denaturation of the protein. Should this be the case, then the change to paraperoxidase is in all probability due to a replacement of the water molecule occupying the sixth co-ordination position by a group in the protein, so that in paraperoxidase two of the six iron valencies are satisfied by the protein. Although paraperoxidase is not a natural product, its properties and the conditions of its formation deserve further study. The term paraperoxidase should therefore be retained for this interesting derivative.

In this connexion, it is of interest to note that similar spectroscopic changes may also occur in solutions of catalase. Thus a solution of horse-liver catalase of Kat.f. = 43,000 was left in a refrigerator for about 2 weeks during which time its activity remained constant. At the end of this period, however, its absorption was considerably modified, the band in the red at 622 mμ. being greatly diminished in intensity while two strong bands appeared at 575 and 537 mμ. This absorption spectrum was not affected on treating the solution with either $Na_2S_2O_4$ or $K_3Fe(CN)_6$, but on addition of NaN_3 the two new bands disappeared and a typical azide-catalase spectrum was formed. On passing a current of air through the solution of modified catalase for several hours the absorption spectrum reverted to that of normal catalase. So far no explanation can be offered as to the nature of these changes in catalase solutions.

Reaction of catalase with hydrogen peroxide

Peroxidatic activity of catalase. The rapid decomposition of H_2O_2 by tissue extracts has been known for more than a century (Thenard, 1818). It was considered at first as a general property of tissues shared only with finely divided metals and later as a property common to all enzymes. It was eventually recognized by several workers and definitely demonstrated by Loew (1901) that this reaction is catalysed by one specific enzyme which he named *catalase*. From that time the decomposition of H_2O_2 to molecular oxygen and water was believed to be the sole function of catalase. However, we have shown (Keilin & Hartree, 1936*b*) that in addition to this reaction catalase is also capable of catalysing the coupled oxidation of the lower alcohols by means of H_2O_2 produced in certain primary oxidation reactions (e.g. those catalysed by xanthine oxidase,

uricase, D-amino-acid oxidase and glucose oxidase). Although for this reaction catalase can also use free H_2O_2, provided it is added slowly and continuously, yet the primary oxidation reaction is by far the most efficient mechanism of supply of H_2O_2 since nearly every molecule of H_2O_2 thus formed can be utilized in the oxidation of alcohols. In all other methods of supply of H_2O_2 only a small fraction is utilized in peroxidatic oxidation, the main portion being decomposed catalatically (Keilin & Hartree, 1945*b*). These observations confirmed our earlier conclusion that 'the physiological function of catalase would be mainly peroxidatic and only in exceptional cases a catalatic one' (Keilin & Hartree, 1936*b*). The peroxidatic activity of catalase has been confirmed and extended by other workers. To the six alcohols: methanol, ethanol, *n*-propanol, *iso*butanol, β-aminoethanol and ethylene glycol, which we previously found acting as hydrogen donors in peroxidatic oxidations catalysed by catalase, Chance (1947, 1948) has added formaldehyde, formic acid, ascorbic acid and peroxides, while Heppel & Porterfield (1949) have added nitrite. What is, however, of special interst is that among hydrogen donors in peroxidatic oxidation by catalase Chance (1948, 1949*d*) includes H_2O_2 itself. In other words, H_2O_2 may act both as substrate and hydrogen donor, the reaction consisting in the oxidation of one molecule of H_2O_2 by another. This would bring even the catalatic decomposition of H_2O_2 into the category of peroxidatic reactions.

Absorption spectra of catalase-peroxide compounds

We have seen that when H_2O_2 is added to methaemoglobin or peroxidase the colour and the absorption spectrum of these haemoproteins undergo marked changes (cf. pp. 88 and 93). On the other hand, when H_2O_2 is added to catalase, the explosive decomposition of H_2O_2 to O_2 and water is not accompanied by any obvious changes in the colour and absorption spectrum of the solution that could be observed in the usual way. Only by applying the rapid flow method and a very sensitive spectrophotometer to the study of this problem was Chance (1947, 1949*b*) able to observe the formation of the primary catalase-peroxide complex, which can be recognized by a slight decrease and a shift towards the red of the Soret band as well as by the presence of a band at about 670 mμ. (Chance, 1950). He was also able to show that the formation of this complex involved only one of the four haematin nuclei of mammalian catalase. Moreover, he considers that 'the attachment of more than one molecule of hydrogen peroxide to catalase results in an as yet undetected complex which rapidly decomposes'. In other words it is the catalatic activity of the enzyme that limits the saturation value of the primary catalase H_2O_2 complex to 1 molecule of H_2O_2 per molecule of catalase.

With H_2O_2 continuously generated by an oxidizing system such as notatin, glucose and oxygen, Chance (1950) observed that the rapidly formed primary catalase-H_2O_2 complex (I) changes gradually into a secondary complex (II) which shows two absorption bands in the visible region: α, 572 and β, 536 mμ. He has studied the kinetics of formation of complex II under various conditions and has suggested that the compound with two absorption bands at 565 and 535 mμ. which is formed when ascorbic acid is added to catalase is not a catalase-ascorbic complex, as was believed by Lemberg & Foulkes (1948), but is in fact the catalase-peroxide complex II, the H_2O_2 arising from autoxidation of ascorbic acid. The variation in the position of absorption bands of complex II with the experimental conditions led us to further studies of the reactions of catalase with continuously generated H_2O_2. The main result of this work was the characterization of a new complex which can be described as catalase-H_2O_2 III.

Experiments with catalase, notatin and glucose. To a small vertical glass tube, 9 mm. diameter, with a fused optical bottom, was added 0·4 ml. catalase (0·184 mM with respect to haematin), 0·4 ml. 0·2 M-acetate buffer pH 5·0, 0·1 ml. 0·5 M-glucose and a trace of octyl alcohol. The tube was stood above the condenser of a microscope and the reaction followed with a microspectroscope. On adding 0·1 ml. notatin solution (0·1 %) and passing a fine stream of air bubbles through the mixture for a few seconds, the colour of the solution turned from brown to red and the absorption bands of free catalase were replaced by two bands: α 568 and β 536 mμ. The positions of these bands were confirmed with a Hartridge reversion spectroscope. If aeration was continued the α-band tended to spread towards the red and thus become asymmetric, while eventually a very feeble band appeared at 585 mμ. On addition of 0·2 ml. 0·1 N-H_2O_2 to the aerated solution, the absorption bands of complex II were replaced by two bands lying nearer to the red: α, 585 and β, 545 mμ. which we ascribe to the complex III. Both II and III revert on standing to free catalase. Experiments at different pH showed that the above reactions proceeded rapidly only within the range 4·0–5·5.

Experiments with catalase and ascorbic acid. It can easily be demonstrated that the changes in colour and absorption spectrum of catalase that are brought about by ascorbic acid are due to the formation of catalase-H_2O_2 II and not to a compound between catalase and ascorbic acid. Thus the reaction requires the presence of oxygen, it is greatly accelerated by the addition of copper salts and the compound obtained is spectroscopically indistinguishable from that observed on aeration of catalase + notatin + glucose.

The reaction was carried out in the vertical glass tubes using 0·4 ml. catalase (as above), 0·4 ml. buffer, 0·1 ml. neutralized ascorbic acid (0·5 M) and various quantities of $CuSO_4$ (0–1 μmole) in 0·1–0·2 ml. water. In absence of copper, aeration of this mixture within the pH range 3·9–6·8 caused no change in absorption spectrum during 5 min. In an experiment at pH 5·0 (0·1 M-acetate buffer) the addition of 0·1 μmole $CuSO_4$ during aeration had no effect, whereas 0·4 μmole gave rise immediately to complex II with bands at 568 and 536 mμ. When the solution was allowed to stand for a few minutes the α-band widened towards the red and appeared to have a maximum at 572 mμ. At this stage the solution was aerated again and a further 0·6 μmole $CuSO_4$ was added. The complex II was immediately and completely transformed into complex III with bands at 586 and 546 mμ. At pH 3·9 a very similar result was obtained, but more ascorbic acid was required for the complete formation of complex III. Under similar conditions at pH 5 the very sparingly ionized copper glycine gave rise to complex II plus a trace of III while even the water-insoluble copper acetylacetone catalysed the slow production of complex II. In all experiments involving ascorbic acid the formation of complexes with H_2O_2 was accompanied by precipitation of catalase. No precipitate was formed if either ascorbic acid or copper was omitted. Nevertheless, even after precipitation the spectra reverted on standing to that of free catalase.

From the above results it is clear that the absorption bands of catalase-H_2O_2 II are: α, 568 and β, 536 mμ., while those of complex III are: α, 585 and β, 545 mμ. The α-band at 572 mμ. previously recorded (Chance, 1950) is probably that of complex II at a stage where it has undergone a slight conversion to III.

Reaction of methaemoglobin (Methb) with hydrogen peroxide

It has been established (Keilin & Hartree, 1935; Haurowitz, 1935) that methaemoglobin forms with H_2O_2 and with ethyl hydroperoxide well defined compounds analogous to those which it forms with cyanide, fluoride, sulphide and azide. The formation of Methb-peroxide requires 1 molecule of peroxide per iron atom of Methb. The Methb-hydrogen peroxide (abbreviated to Methb-OOH) is red in colour and shows two bands: α, 589 and β, 545 mμ. The β-band is stronger than the α-band which is diffuse, asymmetric and may even appear to have two maxima. The complex nature of this band as well as the fact that both peroxidase and catalase form with H_2O_2 several spectroscopically recognizable compounds have prompted us to re-examine more closely the spectroscopic changes which accompany the reaction between Methb and H_2O_2. A preliminary account of this work has already appeared (Keilin & Hartree, 1950*b*).

For this purpose Methb was obtained from catalase-free crystalline horse oxyhaemoglobin by treatment with $NaNO_2$ or with $K_3Fe(CN)_6$ and subsequent dialysis. The experiments were carried out in the small vertical tubes described above and the reactions were followed with a microspectroscope. The solution of Methb used in these experiments was 0·384 mM with respect to haematin and was made up in 0·2 M-phosphate buffer pH 6·8. A 6 mm. depth of this solution clearly showed the absorption band in the red of Methb. The quantity of H_2O_2 added to Methb will be expressed in terms of the molar ratio (R) of H_2O_2 to haematin. When $R=1$ the formation of Methb-OOH was 80 % complete (see also Keilin & Hartree, 1935). When $R=2·5$–5, Methb-OOH was completely formed, but on standing it decomposed liberating acid Methb. During this process part of the Methb underwent destruction.

As the concentration of H_2O_2 was increased the double appearance of the α-band became more marked, the band showing two maxima at 578 and 592 mμ. When $R=25$ the α-band was distinctly double, its two components being of almost equal intensity. When $R=100$ the band at 578 mμ. was much stronger than the band at 592 mμ. When R reached 250–500 the band at 578 mμ. predominated and after a short time the band at 592 mμ. became completely invisible. Thus in presence of an excess of H_2O_2 the Methb-OOH was replaced by another compound which could easily be recognized as oxyhaemoglobin (HbO_2). In fact its absorption spectrum was indistinguishable from that of HbO_2. On treatment with $Na_2S_2O_4$ it gave deoxygenated haemoglobin (Hb) and in presence of CO it formed HbCO. The same sequence of events can be observed by using the notatin system to supply H_2O_2 as described above for the study of catalase-H_2O_2 compounds. On treating a mixture of Methb, notatin and glucose at pH 6·8 with a stream of air Methb-OOH is rapidly formed, but does not become appreciably reduced to HbO_2. Since at the same time the concentration of H_2O_2 remains very low and there is consequently no appreciable destruction of haematin, the maximum at 592 mμ. stands out very clearly with only a faint shading at 578 mμ. representing a trace of HbO_2.

In experiments with H_2O_2 added as a solution it is of special interest that bubbles of O_2 deriving from the decomposition of H_2O_2 do not appear until the α-band of HbO_2 becomes discernible, and active decomposition of H_2O_2 takes place only when the bands of HbO_2 are already very distinct. The reaction between Methb and H_2O_2 therefore consists in the formation of the Methb-OOH complex which, on further reaction with H_2O_2, brings about its decomposition to molecular O_2 and water, a

reaction which is accompanied by the reduction of Methb to Hb and the oxygenation of the latter to HbO_2. The greater the excess of H_2O_2 added from the beginning the more rapid and complete is the formation of HbO_2 and the more vigorous is the decomposition of H_2O_2. But however great is the excess of H_2O_2, the appearance of HbO_2 is always preceded by a transient stage when the total Methb is converted into Methb-OOH. On addition of a suitable hydrogen donor, such as ascorbic acid, which can be oxidized peroxidatically, to a solution showing the absorption bands of HbO_2 and Methb-OOH, the latter is rapidly decomposed liberating Methb while HbO_2 remains temporarily unchanged. The catalatic decomposition of H_2O_2, together with the appearance of HbO_2, are soon followed by a destruction of the haem nucleus as shown by disappearance of absorption bands of the solution which becomes colourless. Since the absolute extinction of the α-band of HbO_2 is much greater than that of Methb-OOH when the relative concentrations of these two compounds in the solution are equal, the α-band of HbO_2 appears to be much stronger than that of Methb-OOH.

All the above reactions have been observed with both recrystallized methaemoglobin and metmyoglobin at pH's varying between 5·5 and 9·2. At pH below 5 the reaction follows a different course. Thus with great excess of H_2O_2 the Methb-OOH formed is rapidly replaced by HbO_2, whereas with smaller concentration of H_2O_2 the Methb-OOH is gradually replaced by a new compound with a wide absorption band at 590 mμ. and showing red-green dichroism. The formation of this compound is preceded by an intermediate stage devoid of any distinct bands in the visible region. However, the addition of NaN_3 or NaF at this stage yields the corresponding Methb derivative, which can readily be identified spectroscopically, while $Na_2S_2O_4$ gives rise to Hb. The haematin prosthetic group, or at least a considerable proportion of it, is thus intact. If the minimum of H_2O_2 is used the compound showing the 590 mμ. band is stable for an hour or more. These conditions are obtained by adding 0·1 ml. 0·05 N-H_2O_2 to 7·5 ml. Methb (0·077 mM with respect to haematin) in 0·2 M-acetate buffer pH 4·5. The 590 mμ. band reaches full intensity after about 8 min. The addition of pyridine and $Na_2S_2O_4$ gives rise to a strong protohaemochromogen with two very faint bands nearer to the red, but the addition of NaF causes only a slow appearance of fluoride-Methb in very poor yield. These results indicate an oxidation of the porphyrin ring, possibly at the methine bridges, without an opening of the tetrapyrrol structure. This view is confirmed by the finding that the Soret band persists, but is much less intense ($\epsilon^{410}_{max.} = 40{,}000$ as compared with $\epsilon^{405}_{max.} = 163{,}000$ for acid Methb).

Since the peroxidatic and catalatic activities of Methb, although much lower than those of peroxidase and catalase, are nevertheless very distinct, Methb can be considered as a suitable model for the study of the reactions catalysed by these two enzymes. As the catalatic decomposition of H_2O_2 by Methb involves the formation of Methb-OOH and subsequent valency changes of Methb iron, it is reasonable to assume that the similar decomposition of H_2O_2 by catalase is also accompanied by valency changes of catalase iron, a view which is, moreover, supported by the study of azide-catalase (Keilin & Hartree, 1936*a*, 1945*a*).

DISCUSSION

Properties of peroxidase

As the quantitative study of different properties, including the absolute absorption spectra, of peroxidase required highly purified enzyme preparations a comparatively simple method was developed for isolation and purification of peroxidase from horse-radish roots. Our most active preparation had a P.N. of about 1220. Its haemin content was 1·61 % which corresponds to a minimum molecular weight of 40,500 (± 600) while sedimentation-diffusion studies carried out by Dr A. G. Ogston and Dr R. Cecil (see Addendum, p. 105) gave the figure 39,800.

Although the haemin content of our purest peroxidase preparation (1·61 %) is higher than the value (1·48 %) given by Theorell (1942*a*), it should perhaps be mentioned that the haemin content of one of his crystalline preparations, of which he obtained 2·8 g., was actually 1·62 %. He concluded, however, that this preparation 'still contained some hemin that did not belong to the peroxidase molecule, which according to the determination of molecular weight should contain 1·48 % haemin'. The high haemin value (1·62 %) he ascribed to the omission of electrodialysis which should remove the extraneous haematin. The fact that a haemin content of 1·48 % was obtained by Theorell after electrophoresis 'performed at a much too high pH value', which reduced the yield of the enzyme from 2·8 to 0·5 g. suggests the possibility that the enzyme of this preparation may have undergone a change involving loss of haematin.

Although our values of haemin content and of molecular weight differ from those of Theorell by only 8–9 % far greater discrepancies appear in the figure for P.N. Whereas our most active preparations had a P.N. of about 1220, Theorell (1942*a*) regards P.N. 930 as the activity of the pure enzyme and ascribes his occasional higher values (1100) to catalytic impurities. We have confirmed the findings of previous workers (summarized by Ettori, 1949) namely, that the values of P.N. obtained in a series of estimations on the same sample under apparently

identical conditions can vary appreciably. In an attempt to make the P.N. estimation more reliable Sumner & Gjessing (1943) carried out the reaction in a small volume of buffer solution. This method was carefully examined by Ettori who also devised a manometric method based upon CO_2 production during the oxidation of pyrogallol but, according to him, neither method is free from the fluctuations of the normal test. In our experience, these fluctuations can be reduced to a large extent by the use of carefully purified pyrogallol (see Methods). Samples of pyrogallol which have not been so purified give rise to P.N. values which may be not only too high but also too low. Under these circumstances the discrepancies between the results of estimations of P.N. in different laboratories are in no way surprising.

One of the main objects of the present investigation was to determine the absolute absorption spectra of peroxidase and its derivatives both for the visible and the violet or Soret regions of the spectrum. It is important to note that in haemoproteins the most significant changes which are in one way or another associated with the iron atom are more clearly reflected in the general pattern of the absorption spectrum in the visible region than in the Soret band. In fact it was through the study of such changes in the visible region that the haematin nature of peroxidase was established and the formation of peroxidase-peroxide, in other words of an enzyme-substrate complex, was demonstrated for the first time (Keilin & Mann, 1937). It is also the existence of absorption bands in the visible region that makes possible the direct spectroscopic study of peroxidase in crude tissue extracts as well as in slices of horse-radish root, a property which will be valuable for the study of the physiological function of peroxidase.

Freezing and cooling to liquid air temperature of peroxidase and its derivatives in solutions or in slices of horse-radish greatly intensifies and sharpens the absorption bands. In this respect peroxidase behaves like other haematin compounds and many other pigments (Keilin & Hartree, 1949, 1950*a*). In slices cooled with liquid air even the band in the red of free peroxidase can easily be seen. The fact that similar cooling of alkaline peroxidase causes its absorption spectrum to change to that of the neutral form has been interpreted by us as indicating that the sixth co-ordination position around the haematin Fe is occupied by a molecule of water and not by a hydroxyl group as was postulated by Theorell & Paul (1944).

There is no evidence that the 'paraperoxidase' of Theorell (1940) occurs naturally in horse-radish roots, although in fact it may be formed in a solution of pure peroxidase which has been allowed to stand. It appears to be a reversible derivative of peroxidase in which the water molecule attached to the Fe atom is probably replaced by a group belonging to the protein.

Peroxidase forms with hydrogen peroxide one green (I) and two red (II and III) compounds. Since the green compound is the first to appear when peroxidase is treated with a small amount of H_2O_2 in absence of hydrogen donors, Theorell (1942*c*, 1947) suggested that this compound is the only catalytically active complex, i.e. the only one of the three which can react directly with hydrogen donors to yield oxidized donors and free peroxidase. However, this has not been confirmed by Chance (1949*c*) in the course of his study of the kinetics of reactions catalysed by peroxidase. Chance has been able to show that the rate of decomposition of the primary green compound (I) is proportional to the rate of formation and decomposition of the secondary red compound (II). The velocity of transition from I to II is greatly accelerated by the presence of a hydrogen donor and can even reach the velocity of formation of the primary compound. In the catalytic activity of peroxidase the complex II is thus the rate-determining Michaelis intermediate and during catalytic activity the enzyme is liberated not from the primary (I) but from the secondary (II) complex. This indicates that both complexes I and II are involved in the catalytic activity of the enzyme.

Comparison of peroxidase, catalase and methaemoglobin

In comparing these three haemoproteins the term methaemoglobin will be used in the widest sense to cover the oxidized or ferric forms of all haemoglobins. Catalase will also be used without distinction as to origin. On the other hand, the term peroxidase will be applied only to the enzyme obtained from horse-radish roots, since the knowledge so far available of other peroxidases, especially of their prosthetic groups, is far from complete.

We shall examine separately (*a*) the properties which are common to these haemoproteins and (*b*) the properties in which they differ.

(*a*) *Properties common to peroxidase, catalase and methaemoglobin*

(1) They are haemoprotein compounds having an identical prosthetic group, protohaematin, united to different proteins. (2) Their iron is in the trivalent state. (3) The colour and the general pattern of their absorption spectra are those of acid methaemoglobin (Fig. 5). (4) All three haemoproteins react reversibly with KCN, H_2S, NaF, NaN_3, NH_2OH, NO, H_2O_2 and ROOH (alkyl peroxides) forming spectroscopically well defined compounds, the formation of which requires 1 molecule of each of these substances per iron atom. (5) The cyanide and fluoride derivatives

of the three haemoproteins show very similar absorption spectra (Figs. 1, 6 and 7). (6) On denaturation or treatment with pyridine and $Na_2S_2O_4$ they give the same haemochromogen. They also yield the same haemin and the same porphyrin (protoporphyrin IX).

(*b*) *Differences in properties of peroxidase, catalase and methaemoglobin*

(1) Although the absorption spectra of these haemoproteins are of the same general pattern, there are certain interesting differences in the position and in the relative heights of their bands (Figs. 1–3, 5–7, also Table 2). The absorption spectrum of catalase in the visible region is represented in Fig. 5 by two curves obtained (i) from liver catalase, which contained a certain amount of verdo-haematin, and (ii) crystalline bacterial catalase, which is completely free from verdo-haematin (from the data of Herbert & Pinsent, 1948). As shown in Fig. 5, the band in the red (α) of catalase is much higher than those of methaemoglobin and peroxidase, the latter being the lowest of the three and lying nearer the red end of the spectrum. On the other hand, the relative heights of their Soret bands (γ) follow the

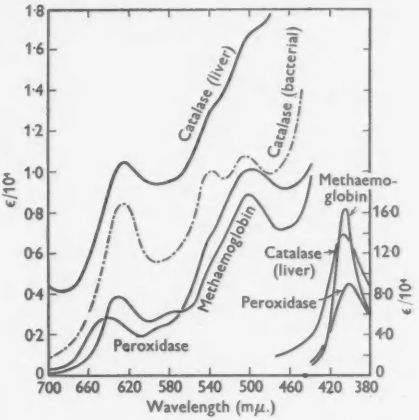

Fig. 5. Absolute absorption spectra of methaemoglobin, peroxidase and liver catalase (present investigation) together with the corresponding curve for bacterial catalase from the paper by Herbert & Pinsent (1948). For definition of units see Fig. 1.

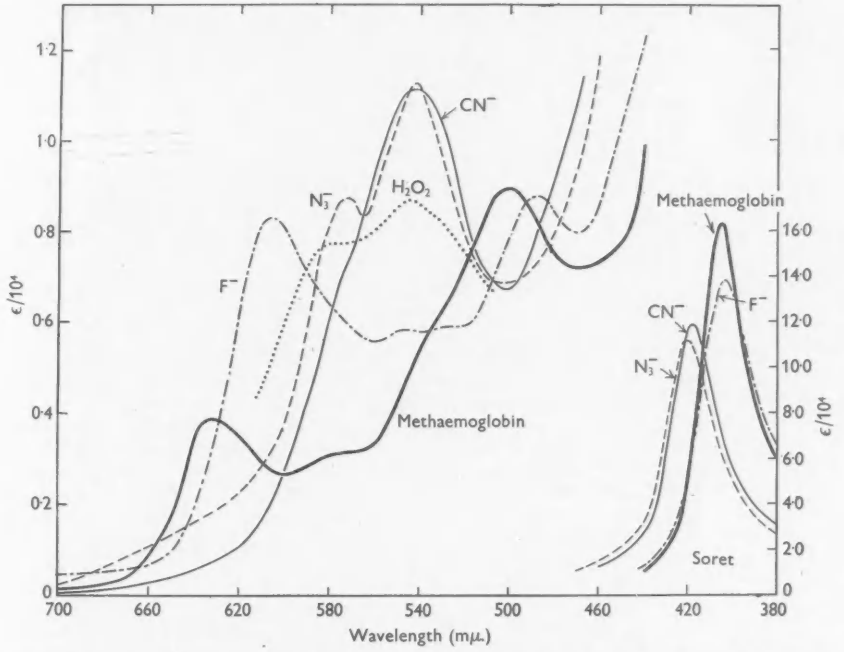

Fig. 6. Absolute absorption spectra at pH 6 of methaemoglobin (prepared from crystalline horse oxyhaemoglobin) and of its fluoride (F^-), cyanide (CN^-), azide (N_3^-) and H_2O_2 derivatives. For definition of units see Fig. 1.

order: Methb > catalase > peroxidase. The ratios of absorption coefficients $\epsilon_\gamma/\epsilon_\alpha$ for these three haemoproteins are: Methb 39, peroxidase 31 and catalase 9–14·2. So far no explanation can be offered for the much stronger absorption of light by catalase and its derivatives in the visible region compared with those of peroxidase and Methb.

unaffected by this reducing agent. In this respect catalase differs from all known haematin compounds.

(4) Methb (Fig. 6) and peroxidase (Fig. 1) on treatment with azide turn from brown to red and their absorption spectra are drastically changed. Catalase (Fig. 7), on the other hand, becomes somewhat more greenish and retains the same general

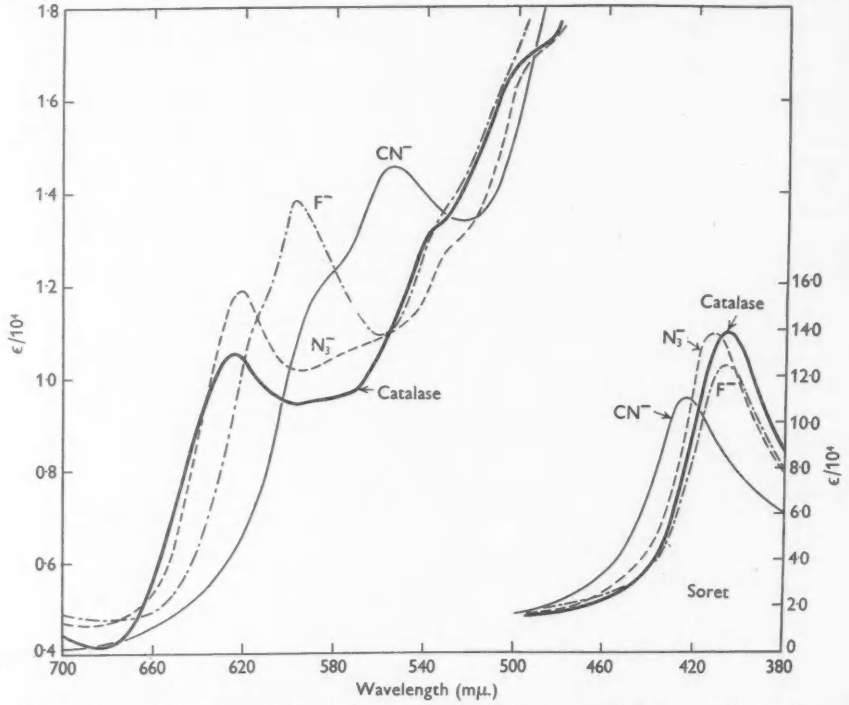

Fig. 7. Absolute absorption spectra at pH 6 of catalase (from horse liver) and of its fluoride (F^-), cyanide (CN^-) and azide (N_3^-) compounds. For definition of units see Fig. 1.

(2) On making a solution of Methb alkaline its colour changes from brown to red and its characteristic absorption spectrum is replaced by two wide bands at 545 and 575 mμ. and a narrow band at 600 mμ. Methb behaves as an indicator between pH 6·8 and 9·5 with pK = 8·1. Peroxidase, like Methb, shows changes in colour and absorption spectrum with changes of pH. At pH 11 a reddish alkaline peroxidase is obtained with bands at 574 and 545 mμ. (Fig. 2). On the other hand, the colour and absorption spectrum of catalase are only slightly affected by changes in pH.

(3) Methb and peroxidase (Fig. 2) are rapidly reduced by $Na_2S_2O_4$ from the ferric to the ferrous state as is shown by changes in their colours and absorption spectra, whereas catalase is completely

spectral pattern, the only noteworthy change being that the band in the red becomes reinforced and shifted slightly towards the blue.

(5) Azide-catalase differs from azide-Methb and azide-peroxidase not only in its optical properties but also in its reaction with H_2O_2. Thus H_2O_2, when added to azide-Methb or azide-peroxidase, partially replaces the azide and forms the corresponding peroxide complexes in which the Fe remains trivalent. On the other hand, H_2O_2 reacts with azide-catalase to reduce its Fe to the divalent state, forming a compound which reacts both with oxygen and with CO. It was the study of this reaction which suggested that the catalatic decomposition of H_2O_2 by free catalase is also accompanied by valency changes of the iron (Keilin & Hartree, 1936*a*, 1945*a*).

Table 3. *Wavelengths (mμ.) of absorption bands in the visible region of complexes formed reversibly by peroxidase, catalase and Methb with* H_2O_2

		Complex II		Complex III	
	Complex I	α	β	α	β
Peroxidase	657	555*	527	583	546
Catalase	670 (?)	568	536	585	545
Methb	Not known	Not known		592*	545

* These bands are asymmetric and the figures given represent the wavelengths of maximum absorption and not the centres of the bands.

(6) The three haemoproteins form with H_2O_2 different complexes which, according to their absorption spectra in the visible region, can be classified as shown in Table 3. Thus peroxidase and catalase give three spectroscopically recognizable complexes of which peroxidase I and II and catalase I appear to be active in reactions which are so far known to be catalysed by these enzymes. Methb forms only one recognizable complex which corresponds to the type III given by peroxidase and catalase. On further reaction with H_2O_2 this Methb complex undergoes reduction to Hb and oxygenation to HbO_2, a reaction which is accompanied by catalatic decomposition of the H_2O_2.

(7) The most striking differences in the properties of these three haemoproteins are found in the magnitude of their catalytic activities. Whereas Methb shows only very slight peroxidatic and catalatic activities, peroxidase and catalase are very powerful catalysts.

For a long time the catalytic activities of peroxidase and catalase were considered to be fundamentally different, peroxidase utilizing H_2O_2 for the oxidation of various substances and catalase being capable only of decomposing H_2O_2 into oxygen and water. However, this difference lost all significance when catalase was shown to be capable of utilizing H_2O_2 for the peroxidatic oxidation of alcohols (Keilin & Hartree, 1936*b*, 1945*b*). The list of hydrogen donors for peroxidatic activity of catalase has since been extended (see p. 97) and now includes H_2O_2 itself (Chance, 1948, 1949*d*). The difference in the catalytic activities of peroxidase and catalase is therefore an expression of their specificity towards hydrogen donors undergoing peroxidatic oxidation.

Although the kinetics of the reactions of catalase and peroxidase with their substrate, and also with hydrogen donors and inhibitors, have been extensively studied the structures of the various enzyme-substrate intermediates remain obscure. Furthermore, in spite of the extensive investigations to which these enzymes have been subjected, work which has led to their complete purification and to a considerable knowledge of their constitution, very little is known of their role in cellular metabolism.

SUMMARY

1. A comparatively simple method is described for the isolation and purification of peroxidase from horse-radish roots.

2. The preparation contained 13·2% nitrogen and 1·61% haemin. The mean value of its activity in terms of Purpurogallin Number was 1220 and the molecular weight was about 40,000 (see Addendum, p. 105).

3. Absorption curves in the visible and Soret regions are given for peroxidase, for its alkaline and reduced forms, and for its compounds with carbon monoxide, cyanide, azide, fluoride and hydrogen peroxide.

4. The absorption bands in the visible region give a better indication than those in the Soret region of changes in the Fe linkages of peroxidase and its derivatives. These bands may be observed in crude enzyme preparations and even in slices of horse-radish root.

5. No evidence was found that 'paraperoxidase' exists as such in horse-radish roots, but it could be obtained as a transformation product of pure peroxidase.

6. Peroxidase catalyses the oxidation of dihydroxymaleic acid directly without the need for quinol or any other 'carrier'.

7. The effect of liquid air temperature on the absorption spectra of peroxidase and methaemoglobin suggests that the sixth co-ordination position around the iron atom in these haemoproteins, at a neutral or slightly acid pH, is a water molecule rather than a hydroxyl group.

8. Peroxidase gives rise successively on addition of hydrogen peroxide to three complexes which are easily recognized spectroscopically. Their absorption bands in the visible region are: I, approx. 657 mμ.; II, 555 and 527 mμ.; III, 583 and 546 mμ. Only I and II appear to take part in the normal catalytic activity of the enzyme. The band which appears at 670 mμ. as a result of prolonged treatment with hydrogen peroxide is probably that of a degradation product of peroxidase rather than a complex with hydrogen peroxide.

9. Catalase also forms with hydrogen peroxide three spectroscopically recognizable complexes with

the following bands: I, approx. 670 mμ.; II, 568 and 536 mμ.; III, 585 and 545 mμ. Complex I, which can only be detected by the rapid-flow technique, appears to be the only one that participates in the known reactions of this enzyme. Complex II can be obtained when hydrogen peroxide is supplied continuously by an oxidizing system; it can be converted to III by further reaction with hydrogen peroxide.

10. Methaemoglobin forms only one complex with hydrogen peroxide. The bands are at 592 and 545 mμ. and it thus corresponds to type III of peroxidase and catalase. The reaction of this complex with further hydrogen peroxide results in the reduction of methaemoglobin to haemoglobin, which can be detected as oxyhaemoglobin, and simultaneous liberation of oxygen from hydrogen peroxide.

11. Peroxidase resembles catalase and methaemoglobin in structure (all three being protein-protohaematin compounds), colour, general pattern of absorption spectra and formation of reversible and spectroscopically recognizable compounds with cyanide, sulphide, azide, fluoride, nitric oxide and peroxides.

12. Peroxidase and methaemoglobin, unlike catalase, can be reduced with sodium dithionite and show marked changes in colour and absorption spectrum with pH or on combining with azide. Azide-catalase, however, is spectroscopically very similar to free catalase and is reduced to a ferrous derivative by hydrogen peroxide.

13. There are no fundamental differences in the nature of the catalytic activities of peroxidase and catalase since the latter has been shown to catalyse peroxidatic oxidation of alcohols and other substances. However, the peroxidatic activity of catalase shows maximum efficiency only when hydrogen peroxide is continuously supplied by a system which reduces oxygen to hydrogen peroxide.

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ADDENDUM

Determination of Sedimentation and Diffusion Constants of Horse-Radish Peroxidase

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(*Received* 13 *September* 1950)

Samples of peroxidase (fraction *H*), provided by Prof. D. Keilin and Dr E. F. Hartree were examined in a Svedberg oil-turbine ultracentrifuge and in the Gouy diffusiometer (Coulson, Cox, Ogston & Philpot, 1948). The buffer was NaCl, 0·2M; KH_2PO_4, 0·046M; Na_2HPO_4, 0·010M.

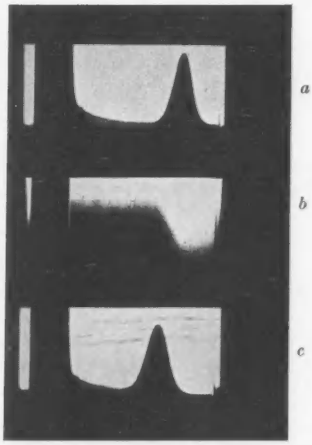

Fig. 1. Sedimentation diagrams: 40, 65 and 80 min. after reaching full speed; *a* and *c* by refraction, and *b* by absorption.

The sedimentation constant was first determined in a solution containing 7·7 mg./ml. at 1000 rev./sec.; observations were made alternately by the diagonal-schlieren method of Philpot (1938) at 600–640 mμ., and by absorption (Philpot, 1939) at 540–570 mμ. Fig. 1 shows refractometric and absorptiometric diagrams. In Fig. 2 the positions of the main boundary at successive times are shown plotted as $S(\Delta x)$ against $S(\Delta t')$ (Cecil & Ogston, 1948): the positions obtained from absorption lie closely on the same curve as those obtained refractometrically, showing that the absorbing material sediments exactly with the main refracting component. Of the total refracting material 92% forms the main sedimenting boundary (Cecil & Ogston, 1948), the remaining 8% being probably accounted for by a small amount of heterogeneous material. A second determination of the sedimentation constant, at a concentration of 3·4 mg./ml., was made for comparison with the diffusion constant determined at the same concentration.

Diffusion was observed by the Gouy method, using light of 622 mμ. (from a mercury lamp). At 6·9 mg./ml. the optical extinction of the solution was 0·37 and this proved to be too great for a really satisfactory record to be obtained: however, the result indicated that the material is nearly homogeneous with respect to diffusion. At 3·4 mg./ml. (extinction = 0·18) a satisfactory record was obtained. The theory of the interference of light in the Gouy method (Kegeles & Gosting, 1947) applies strictly only to the case where no absorption of light takes place: elementary theory (which may not be reliable) suggests that a moderate degree of absorption should not result in a large shift of the interference minima, and the results have been interpreted on this assumption.

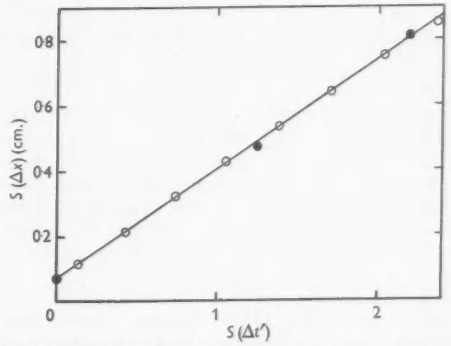

Fig. 2. Plot of $S(\Delta x)$ in cm. measured on the record against $S(\Delta t')$ in units of 10^{12}: ○, by refraction; ●, by absorption.

The sedimentation and diffusion constants (corrected to their values for water at 20°) and other quantities are given in Table 1. The value of the partial specific volume was measured by Keilin & Hartree (1951) and agrees moderately well with the value 0·690 given by Theorell (1942).

Table 1. *Sedimentation and diffusion constants of peroxidase*

Concentration (mg./ml.)	S_{20} (corr.) ($\times 10^{13}$)	D_{20} (corr.) ($\times 10^{7}$)	$\bar{V}$	M	f/f_0	Proportion in main boundary
7·7	3·47	—	—	—	—	0·92
3·4	3·48	7·05	0·699	39,800	1·36	—

Theorell (1942) has reported sedimentation and diffusion measurements on peroxidase: our value for S_{20} (corr.) is about 10 % lower than his ($3{\cdot}85 \times 10^{-13}$) (cf. Cecil & Ogston, 1948), while our value for D_{20} (corr.) agrees fairly well with his value of $6{\cdot}84 \times 10^{-7}$. Our value for the molecular weight is lower than Theorell's (44,100), but is in agreement with that calculated by Keilin & Hartree (1951) from the haematin content; the chief uncertainty in our quantity probably lies in the value of $\bar{V}$.

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Studies on the Enzymic Synthesis of Glutamine

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(*Received* 5 *September* 1950)

Earlier reports described the discovery of an enzyme system in sheep brain which synthesizes glutamine in the presence of L-glutamic acid, adenosinetriphosphate (ATP) and ammonia (Elliott, 1948; Elliott & Gale, 1948). The present paper deals with the purification and detailed study of this enzyme system and the results of a distribution survey. A summary of this work was presented at the First International Congress of Biochemistry at Cambridge in August 1949 (Elliott, 1949). Speck (1947, 1949*a*, *b*) has independently reported a study of the glutamine-forming system present in pigeon liver.

MATERIALS

ATP was prepared as described by Needham (1942) except that in the final stages the method of Kerr (1941) was adopted to produce the monobarium salt. This was converted to the Na salt by dissolving it in N-HCl and adding a slight excess of Na_2SO_4. After centrifuging, the supernatant was neutralized to pH 6·8 and stored at −10°. The solution used contained 0·1 mg. inorganic P/ml. and 4·3 mg. of P hydrolysed by boiling N-HCl in 10 min. (acid-labile P). The solution was analysed enzymically by the method of Bailey (1949). The percentages of the acid-labile P hydrolysed by myosin, by myosin + myokinase, and by inorganic pyrophosphatase were 45·6, 91·2 and 2·6 respectively.

Adenosinediphosphate (ADP) was prepared from ATP by the method of Bailey (1942). The Ba salt was treated in the same way as that for ATP. The solution used contained 3·16 mg. of total P/ml., of which 50 % was hydrolysed by boiling N-HCl in 10 min. The percentages of the total P split by myosin alone, by myosin + myokinase, and by inorganic pyrophosphatase were 1·0, 47 and 0·02 respectively.

Adenylic acid (AA) was supplied by the Armour Laboratories, Chicago.

L-Glutamic acid hydrochloride, hydroxylamine hydrochloride and hydrazine sulphate were commercial samples twice recrystallized from water. All solutions were neutralized before use.

Methylamine hydrochloride was prepared free from NH_4Cl by the method of François (1907). A large excess of yellow HgO was used to make sure that all traces of NH_3 were removed.

DL-Methionine oxide was prepared by the method of Toennies & Kolb (1939).

D-Glutamic acid, *N*-carbobenzyloxy-L-glutamic acid, L-*iso*glutamine (L-glutamic acid α-amide), and L-glutamine were kindly given by Dr A. C. Chibnall.

Phosphate buffers were mixtures of Na_2HPO_4 and KH_2PO_4.

Glyoxaline buffers were prepared by adjusting the pH of solutions of the base with HCl.

METHODS

Incubations were usually performed in open tubes at 30° and samples taken as necessary for estimations.

Chemical determinations

Hydroxamic acid was estimated by the method of Lipmann & Tuttle (1945) except that determinations were done on 4·5 ml. samples as described previously (Elliott & Gale, 1948).

Ammonia. Samples were deproteinized by mixing with 0·2 vol. of 25% (w/v) aqueous trichloroacetic acid and centrifuging. The supernatant was neutralized to pH 6·8 and a suitable portion taken for distillation of NH_3 in the Parnas & Heller apparatus. The NH_3 in the distillate was determined colorimetrically after nesslerization.

Glutamine was estimated according to the method described by Krebs (1935).

Phosphate was estimated by the method of Fiske & Subbarow (1925).

Elementary analyses were by Drs Weiler and Strauss of Oxford.

Preparation of enzyme-containing extracts

Purification of the glutamine-synthesizing system of sheep brain. The standard test system for estimating enzymic activity contained the following mixture: 0·2 M-glyoxaline buffer (pH 7·1), 1·0 ml.; 1·0 M-sodium L-glutamate, 0·5 ml.; sodium adenosinetriphosphate (4·0 mg. P 10/ml.), 1·0 ml.; 1·0 M-neutralized $NH_2OH.HCl$, 0·2 ml.; 1·0 M-$MgCl_2$, 0·2 ml.; enzyme solution + water, 1·4 ml. The tubes were incubated at 30° for 15 min. and the hydroxamic acid was then estimated. Under these conditions the hydroxamic acid production was proportional to the amount of enzyme added up to a value of about 4 μg.mol. hydroxamic acid. It was not possible at this stage to use the more accurate method of estimating enzymic activity by means of phosphate liberation from ATP owing to the high adenosinetriphosphatase (ATPase) activity in the crude extracts.

Acetone-dried grey matter of sheep brain was extracted with 10 vol. of distilled water for 10 min. with very gentle stirring. After centrifuging, the supernatant was filtered through cotton wool, cooled to 4° and mixed with 0·2 vol. of 0·1 M-sodium acetate buffer (pH 4·2). The precipitate was centrifuged down, washed twice by suspending in cold distilled water and centrifuging, and finally redissolved in water (half the original volume of extract) by adjusting the pH to 6·8 with a little NaOH. This gave a fourfold purification on a nitrogen basis with a recovery of 70%. The extract was almost completely free from ATPase activity. Further purification up to 19-fold was achieved by heating the solution in the presence of 0·2 M-acetate buffer (pH 4·4) and 0·1 M-$MgCl_2$ at 43° for 3 min. The precipitate was removed by centrifuging and the enzyme adsorbed on to calcium phosphate gel, followed by elution with 0·2 M-phosphate buffer (pH 7·2).

The stability of the enzyme appeared to decrease as purification proceeded and for this reason it was convenient to use the enzyme at the first stage of purification; this was stable for 2 weeks when stored at 0°.

Bacterial extracts. Extracts of *Staphylococcus aureus* were prepared as described previously (Elliott & Gale, 1948). Extracts of baker's yeast were obtained in the same manner.

Kidney extracts. Acetone-dried rabbit kidney was extracted and purified in the same way as described for brain.

Mealworm extracts. Mealworms (*Tenebrio molitor*) were ground with distilled water in a mortar and the thick suspension centrifuged. The cloudy supernatant was used in experiments.

Plant extracts. Seeds of *Lupinus albus* and *L. angustifolius* were soaked in distilled water and kept to germinate in moist peat for 3–5 days. The seedlings were washed, the seed coats removed and the whole seedlings ground with sand in a mechanical mortar. The mush was squeezed through muslin and the extract centrifuged. The turbid supernatant was dialysed against dilute phosphate buffer (pH 7·1).

Adenylic acid deaminase. The preparation used consisted of a freeze-dried preparation of the fraction precipitated from a dialysed rabbit-muscle extract on addition of 0·1 vol. of ethanol (Askonas, 1950). I am grateful to Miss I. A. Askonas for this preparation.

RESULTS

Enzymic synthesis of glutamine by brain extract

Fig. 1 shows the formation of glutamine by the purified brain extract in the presence of ATP, L-glutamate, ammonia and Mg^{++}. The synthesis is

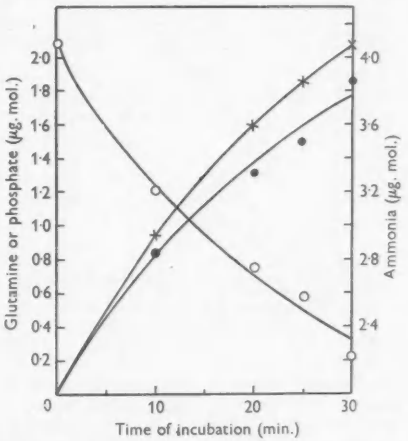

Fig. 1. Synthesis of glutamine from L-glutamate and ammonia by extract of sheep brain. ●—●, Glutamine formed; ×—×, inorganic phosphate; ○—○, free ammonia.

accompanied by the disappearance of free ammonia and the liberation of phosphate from ATP. The ratio of phosphate liberated to amide formed is about 1·1; this may be due to a slight hydrolysis of glutamine during estimation.

Isolation of glutamine

A large-scale experiment was performed so that the glutamine formed could be identified by isolation. The mixture contained 0·04 M-phosphate buffer (pH 7·0); 0·1 M-sodium L-glutamate; 0·02 M-magnesium chloride; 0·014 M-ATP; 0·02 M-ammonium chloride; 0·2 g. purified myokinase; 0·1 g.

(approx.) of a muscle AA-deaminase preparation; 70 ml. of purified brain extract. The total volume was 225 ml. Myokinase and AA-deaminase were added to remove the ADP which is formed during the synthesis of glutamine. The mixture was incubated at 30° in the presence of a few drops of toluene for 20 hr. After incubation glutamine was isolated by the method of Vickery, Pucher & Clark (1936) from the stage of adding lead acetate. The product was obtained as colourless crystals and recrystallized twice from 66% (v/v) aqueous ethanol. This gave 0·35 g. of a product which contained 71% glutamine

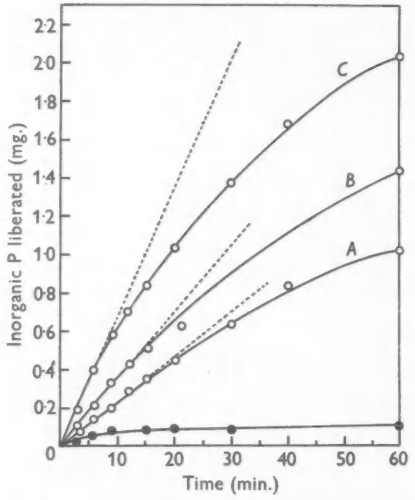

Fig. 2. Progress curves of glutamine synthesis in the presence of different concentrations of enzyme. - - - -, Initial velocities. Volumes of enzyme solution added: control and curve *C*, 1·0 ml.; curve *A*, 0·33 ml.; curve *B*, 0·5 ml. ●—●, Control without glutamic acid. Curves *A*, *B* and *C* have been corrected by subtraction of the control values.

as estimated by amide N. (Found: C, 38·4; H, 6·5; N, 16·5; acid-labile amide N, 6·9. Calc. for $C_5H_{10}O_3N_2$: C, 41·1; H, 6·9; N, 19·2; amide N, 9·5%.) The analysis suggests that some of the impurity was glutamic acid (calculated N for such a mixture is 16·4%). Analysis by paper chromatography showed the presence of glutamine and a smaller amount of glutamic acid. When mixed with glutamine isolated from sugar beet by the method of Vickery *et al.* (1936) only the same two spots appeared on the paper. The preparation was free from ammonia; a solution gave no initial colour with Nessler reagent, but the colour slowly developed as described by Archibald (1945) for glutamine.

Properties of the brain enzyme system

Measurement of activity. The liberation of inorganic phosphate from ATP in the presence of L-glutamate and ammonia was chosen as the most accurate and convenient method of determining enzyme activity. The complete incubation mixture contained the following final concentrations: 0·04M-glyoxaline buffer (pH 7·1); 0·02M-magnesium chloride; 0·1M-sodium L-glutamate; 0·014M-sodium salt of ATP; enzyme solution, 1 ml. The final vol. was 10 ml. Unless otherwise stated, the above conditions were used in all subsequent experiments and

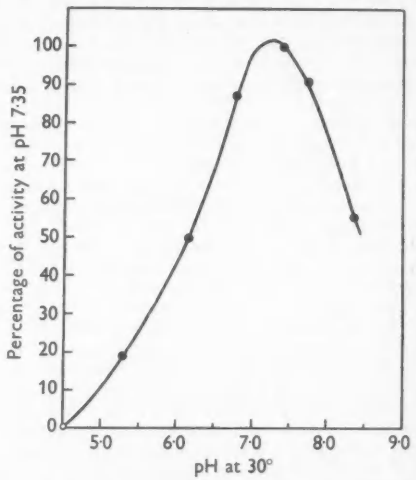

Fig. 3. The effect of pH on the rate of glutamine synthesis. Glyoxaline buffer was used for all except the lowest pH where acetate was used.

only variations of this will be described. Control experiments in which glutamate was omitted from the incubation mixture were carried out in each case to obtain the corrections necessary for ATPase activity; these control values were never more than about 5% of the maximum enzyme activity with respect to glutamine synthesis. All curves shown in the figures are corrected for ATPase activity though in some cases the latter is shown for comparison. Fig. 2 shows the progress of the reaction in the presence of different amounts of enzyme. In the absence of glutamate the increase in phosphate was very small. With the complete system, liberation of phosphate was linear only until 0·04 mg. P/ml. had been liberated, after which the reaction velocity decreased. Since this represents less than 10% breakdown of the ATP present, the decreased rate of reaction cannot be accounted for by exhaustion of substrate. The slopes of the linear portions of the

curves are proportional to the amount of enzyme added and in all subsequent experiments care was taken to ensure that activity measurements fell on the linear part so that the true initial reaction velocity was obtained.

Effect of pH. The optimum pH for the enzymic synthesis of glutamine lies at about 7·2 (Fig. 3).

Effect of substrate concentrations. The activity of the enzyme system increases with higher concentrations of ATP (Fig. 4). The effect appears to be more complex than that of a simple enzyme-substrate relationship, for the higher levels of ATP appear to act by maintaining the linear rate of reaction for a longer period of time rather than by increasing the initial rate of reaction, even though the fall in reaction velocity occurs when only a small proportion of the ATP has been used. This effect is more clearly seen when the curves in Fig. 4 are compared with those obtained with varying levels of glutamate (Fig. 5). In the latter the initial rates of

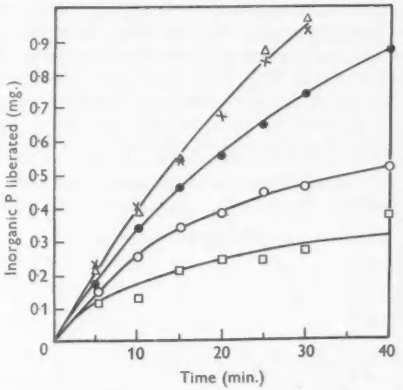

Fig. 4. The effect of concentration of adenosinetriphosphate on the rate of glutamine synthesis. □—□, 0·0017 M; ○—○, 0·0035 M; ●—●, 0·007 M; ×—×, 0·014 M; △—△, 0·026 M.

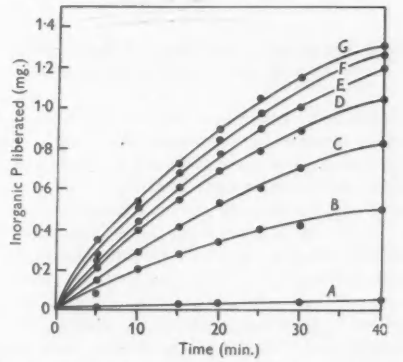

Fig. 5. The effect of glutamate concentration on the rate of glutamine synthesis. The concentrations of sodium L-glutamate used for curves *A–G* were 0, 0·002, 0·005, 0·01, 0·025, 0·05 and 0·1 M, respectively.

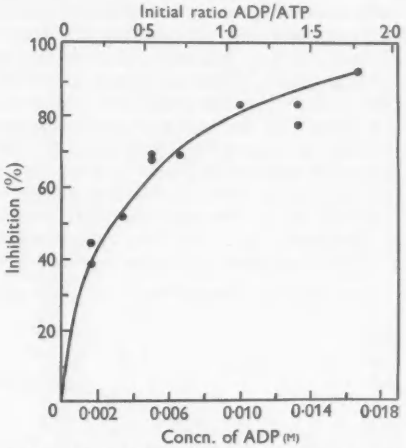

Fig. 6. Inhibition of the synthesis of glutamine by adenosinediphosphate. The concentration of ATP in all experiments was 0·0093 M.

Table 1. *The effect of adenosinetriphosphate (ATP) concentration on the inhibition by adenosinediphosphate (ADP) of glutamine synthesis by sheep-brain extract*

(Incubation mixture as described in text.)

Concn. of ADP (M)	Concn. of ATP (M)	P liberated in 10 min./10 ml. of incubation fluid (mg.)	Inhibition (%)
0	0·005	0·27	—
0	0·009	0·29	—
0	0·019	0·29	—
0·0034	0·005	0·10	64
0·0034	0·007	0·13	55
0·0034	0·009	0·16	45
0·0034	0·012	0·17	41
0·0034	0·014	0·14	(48)
0·0034	0·019	0·17	41

Table 2. *Inhibition of glutamine synthesis in sheep-brain extract by adenylic acid (AA)*

(Incubation mixture as described in text.)

Concn. of AA (M)	Concn. of ATP (M)	P liberated in 10 min./10 ml. of incubation fluid (mg.)	Inhibition (%)
0	0·014	0·29	—
0·01	0·014	0·24	17
0·04	0·014	0·23	20

reaction change with substrate concentration, but the overall shapes of the curves remain unchanged. This effect of ATP concentration on the shape of the progress curve is probably due to the higher concentrations overcoming the inhibition by the ADP which is formed during the synthesis of glutamine. As shown in Fig. 6, ADP is a very powerful inhibitor of the brain system. When the ratio of ADP/ATP is 0·3 a 50% inhibition is obtained. The inhibition by ADP is reduced by increasing the concentration of ATP (Table 1) suggesting competitive inhibition. AA has only a small effect (Table 2); even when the AA/ATP molecular ratio exceeds 1·0 the activity falls by only 20%. This small inhibition could be due to the formation of ADP from AA and ATP by traces of myokinase in the enzyme preparation.

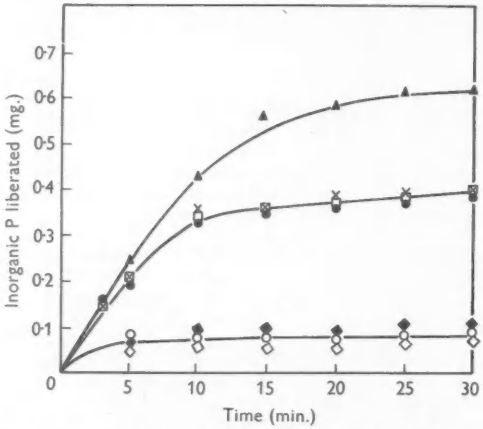

Fig. 7. Enzyme activity in the presence of various amino compounds. ○—○, Control without added base; ◆—◆, 0·02 M-methylamine; ◇—◇, 0·001 M-methylamine; ×—×, 0·001 M-ammonium chloride; ●—●, 0·001 M-L-hydroxylamine; □—□, 0·001 M-hydrazine; ▲—▲, 0·0015 M-ammonium chloride.

Specificity towards L-*glutamate*. D-Glutamate, L-*iso*glutamine, L-glutamine, *N*-carbobenzyl oxy-L-glutamate, L-aspartate and nicotinic acid were all inactive in this system.

Specificity towards '*glutamyl acceptors*'. Fig. 7 shows the results of a series of experiments in which the effect of replacing ammonia by other bases was studied. In the presence of glutamate, but without added ammonia, a small liberation of phosphate occurred during the first 7 min. This increase was approximately equivalent to the amount of free ammonia present as a contaminant in the enzyme preparation (about 0·3 μg. mol./ml.). With the addition of 0·001 M-ammonium chloride, phosphate liberation proceeded rapidly until the increase in phosphate was equivalent to the ammonia added. With half as much again of ammonium chloride (0·0015 M), the initial rate of reaction was about the same, but the final level of phosphate reached was higher. When ammonium chloride was replaced by 0·001 M-hydroxylamine or hydrazine the reaction curves were the same as that with the same concentration of ammonium chloride. The addition of methylamine, even in concentrations as high as 0·02 M, caused no significant increase in phosphate production, showing that this compound does not condense with glutamic acid in this system. This is in contrast to the result obtained by Speck (1949*b*) who found methylamine to react at about the same rate as ammonium chloride in the glutamine-synthesizing system of pigeon liver. This may be due to different specificities of the systems from different tissues, though it must be pointed out that Speck used commercial samples of methylamine which may contain sufficient ammonium chloride to react in the presence of the enzyme system. As shown in Fig. 7, methylamine does not inhibit the reaction with the residual ammonia present in the enzyme preparation. From the progress curves a rough estimate of the K_m towards ammonia, hydroxylamine and hydrazine gives a value of 0·0005 M. The following amino compounds were inactive as glutamyl acceptors: ethanolamine, urea, glycine, glycine ethyl ester, *p*-aminobenzoic acid, aniline, phenylhydrazine, β-phenyl*iso*propylamine and L-glutamine.

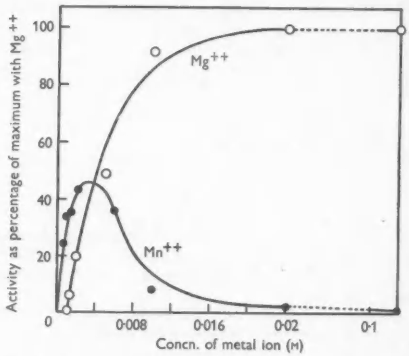

Fig. 8. The effect of concentration of Mg^{++} and Mn^{++} ions on the enzymic synthesis of glutamine.

Effect of metallic ions. The purified brain enzyme system is completely inactive in the absence of Mg^{++} or Mn^{++}. Mg^{++} gives maximal activation at 0·02 M (Fig. 8); at low concentrations Mn^{++} is as effective as Mg^{++}, but at concentrations of Mn^{++} greater than 0·003 M the activity falls rapidly (Fig. 8). The maximal activation by Mn^{++} is only 46% of that given by 0·02 M-Mg^{++}.

Ca^{++} strongly inhibits the synthesis of glutamine, but this inhibition is reduced by increasing the concentration of Mg^{++} (Table 3) suggesting that the Ca^{++} competes with the Mg^{++} in the enzyme system.

Table 3. *The effect of* Mg^{++} *on the inhibition of glutamine synthesis in brain extracts by* Ca^{++}

(Incubation mixture as described in text.)

Concn. of Mg^{++} (M)	Concn. of Ca^{++} (M)	P liberated in 10 min./10 ml. of incubation fluid (mg.)	Inhibition (%)
0·01	0	0·36	—
0·01	0·01	0·024	93
0·02	0·01	0·037	90
0·10	0·01	0·16	56

Inhibitors. Fluoride is the most powerful inhibitor so far found for the brain glutamine-synthesizing system; 50% inhibition is given by 5×10^{-5}M-sodium fluoride (Fig. 9). *p*-Chloromercuribenzoate

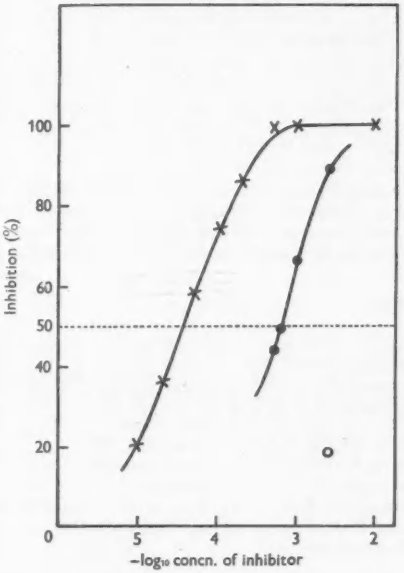

Fig. 9. Inhibition of the glutamine-forming system by fluoride and *p*-chloromercuribenzoate. ×—×, Fluoride; ●—●, *p*-chloromercuribenzoate. The single open circle shows the effect of adding 0·02M-cysteine before adding the *p*-chloromercuribenzoate.

inhibits 50% at a concentration of 10^{-3}M, though this inhibition is largely abolished by the prior addition of cysteine (Fig. 9). In the absence of inhibitor cysteine has no activating effect and, in fact, over long periods of incubation has a slight inhibitory action which may be due to the formation of cystine by oxidation.

The sulphoxide derived from methionine, which inhibits the glutamine-forming system of *Staphylococcus aureus* (Elliott & Gale, 1948), also inhibits the brain preparation, but the latter is about ten times less sensitive (Fig. 10).

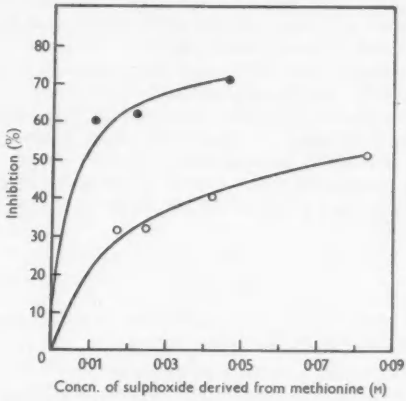

Fig. 10. Inhibition of glutamine synthesis in extracts from sheep brain and *Staphylococcus aureus* by the sulphoxide derived from methionine. ●—●, *Staph. aureus*; ○—○, brain.

Crystal violet, which completely inhibits the bacterial system (Elliott & Gale, 1948), has no effect on the brain system even at a concentration as high as 0·004M.

Distribution. The formation of a hydroxamic acid in the presence of L-glutamate, ATP and hydroxylamine was used as a test for the presence of the

Table 4. *Synthesis of glutamyl-hydroxamic acid by extracts of* Lupinus albus *seedlings*

(For details of incubation mixture, see text.)

Incubation mixture	μg.mol. hydroxamic acid formed from L-glutamate	L-aspartate
Complete system	4·1	0·2
No ATP	0·1	—
No glutamate	0·3	—
No magnesium	0·2	—
With 0·001M-NaF	0·1	—
Boiled enzyme	0·1	—

glutamine-synthesizing system. Positive results were obtained with cell-free extracts of baker's yeast, rabbit kidney, mealworms (*Tenebrio molitor*) and seedlings of *L. albus* and *L. angustifolius*. The activity/ml. in the plant extracts was higher than that in crude brain extracts. The plant system resembled that of animal tissues in being activated

by Mg^{++} and inhibited by low concentrations of fluoride (Table 4). It was not possible to detect any formation of a hydroxamic acid when glutamate was replaced by L-aspartate.

Tests for intermediate compounds in the synthesis of glutamine. It was not possible to detect any formation of an acyl phosphate when no 'glutamyl acceptor' was added to the incubation medium. Addition of hydroxylamine after the enzyme was incubated with glutamate and ATP and then inactivated by the addition of 0·005 M-sodium fluoride did not give a hydroxamic acid. As shown previously there was no liberation of phosphate from ATP unless a glutamyl acceptor such as ammonia was present. Highly labile phosphate derivatives such as acyl phosphates would appear as inorganic phosphate in the estimation by the Fiske & Subbarow method.

DISCUSSION

The results show that glutamine is synthesized according to the following equation:

glutamate + ATP + ammonia
→ glutamine + ADP + phosphate.

It is not yet certain whether one or more enzymes are involved in this reaction. Possibly a transfer of phosphate from ATP to glutamic acid occurs first and the resulting γ-glutamyl phosphate then reacts with ammonia to form glutamine and inorganic phosphate. So far, however, it has not been possible to demonstrate such an intermediate compound.

The discovery of a very active glutamine-forming system in typical 'asparagine type' plants is surprising and gives additional support to the suggestion by Street (1949) that the classical conception of asparagine and glutamine plants adopted by Schultze is no longer tenable. Presumably the glutamine is rapidly used in the asparagine plants since little is found to accumulate (Chibnall, 1939).

SUMMARY

1. The purification of an enzyme system from sheep brain which synthesizes glutamine from glutamate and ammonia in the presence of adenosinetriphosphate (ATP) is described. Inorganic phosphate is liberated in the reaction.

2. The system is specific towards L-glutamate and ATP. Ammonia can be replaced by hydroxylamine and hydrazine but not by organic bases. No reaction occurs in the absence of such 'glutamyl acceptors'.

3. The system is activated by Mg^{++} and Mn^{++}. Higher concentrations of the latter inhibit.

4. Ca^{++}, adenosinediphosphate, the sulphoxide from methionine, fluoride and *p*-chloromercuribenzoate inhibit the synthesis of glutamine.

5. The system has been found in extracts from lupin seedlings. It has not been possible so far to demonstrate an analogous asparagine-forming system.

The author wishes to thank Prof. M. Dixon, F.R.S., and Dr E. C. Webb for the valuable advice and criticism which they have given during the course of this work. Thanks are also due to the Medical Research Council for a personal grant.

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On the Mechanism of the Inhibition of Glycolysis by Glyceraldehyde

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Since the discovery by Mendel (1929) of the inhibition by 0·001 M-glyceraldehyde (GA) of lactic acid formation from glucose in tumour slices, the mechanism of this inhibition has been the subject of much experimentation and discussion. Very varied results are recorded by different workers using slices or brei from various tissues, or tissue extracts with or without added yeast hexokinase; on the whole, the conclusion may be drawn that with systems using glucose, the glycolysis is very sensitive to GA while with glycogen or starch as source, the inhibition arises only with much higher concentrations of GA. The rather numerous exceptions to this rule are probably to be explained on the following grounds: (*a*) only monomeric GA inhibits glucose breakdown. This was not realized before the work of Lehmann & Needham (1938), and earlier workers may have used one form or the other or a mixture of both. Lehmann & Needham also found that a high concentration of the dimeric form ($3{\cdot}0$–$6{\cdot}7 \times 10^{-2}$ M) inhibits phosphorylase. (*b*) Stickland (1941), using muscle extract reinforced with yeast hexokinase, found that the inhibition of glucose breakdown by about 0·003 M-GA can be prevented by a small excess of hexokinase. In earlier work the need for this strict control of the amount of added hexokinase was not understood.

Stickland (1941), taking account of both these precautions, made a careful study of the glycolysis of both starch and glucose, in the presence of GA, in muscle extract. The systems differed only in that with glucose the minimal amount of hexokinase necessary to obtain a steady glycolysis was added. Using only 0·003 M-GA, 85–95 % inhibition was found with glucose or fructose, while 0·03 M-GA was needed with starch to obtain even 5–30 % inhibition. Since the enzymes acting in the two cases are the same with the exception of the phosphorylase and the hexokinase concerned in the initial phosphorylations, Stickland concluded that GA must affect the phosphorylation of glucose by hexokinase. However, later experiments (Stickland, 1942), designed to test directly the effect of GA upon yeast hexokinase, showed no inhibition even with GA concentrations as high as 0·05 M.

After the discovery by Warburg & Christian (1939) and by Dixon & Caputto (1945) that GA can act as a substrate for the triosephosphate dehydrogenase (TPD) of yeast and muscle, but is acted upon much more slowly than glyceraldehydephosphate (GAP) (at about $\frac{1}{300}$th of the rate in the case of muscle), the suggestion arose that GA might inhibit the oxidation of GAP by competing with it for the enzyme. In tissues or in a system such as that used by Stickland, where only catalytic amounts of adenosinetriphosphate (ATP) are present, glycolysis depends upon continual rephosphorylation of the adenosinediphosphate (ADP) formed; inhibition of TPD will interfere with this process. The need for this replenishment of ATP is twice as great when glucose rather than glycogen is the substrate; in the presence of adenosinetriphosphatase, as in muscle extract, the effect of even partial inhibition of TPD might well be to diminish or stop the phosphorylation of glucose through lack of ATP.

The possibility that GA can inhibit TPD in this way was tested by two lines of approach (*a*) indirectly by experiments using the same system as Stickland; (*b*) directly by experiments on pure TPD. As will be described, good evidence was obtained for this inhibition, particularly by the D-form of the aldehyde.

When this work was nearing completion a paper appeared by Rudney (1949) describing the inhibition by L-glyceraldehyde of the hexokinase of various tissues and extracts. In view of the discrepancy between these observations and those of Stickland, the question of hexokinase inhibition by GA was further examined; the degree of inhibition with muscle hexokinase was found to depend on the concentration of sugar present.

EXPERIMENTAL

Tissue extracts and enzyme preparations. (*a*) Rabbit-muscle extract (Meyerhof, 1926) was used for all the glycolysis experiments. The cooled muscle was minced and extracted with 1·5 times its weight of ice-cold water. After standing at 0° for 30 min., the mass was strained through cheese cloth. The filtrate was dialysed in cellophan tubing for 20 hr. against 20 vol. distilled water at 4°. The resulting cloudy fluid (a bulky precipitate appeared during dialysis and its removal led to great loss of activity) was used without centrifugation, being well mixed before samples were taken; it was stored at 4° and kept its activity during several days.

(*b*) The hexokinase was prepared from baker's yeast by the method of Meyerhof (1927). It was precipitated by 50 % (v/v) ethanol, dried, and stored in a desiccator. For the

solution, which was made up fresh each day, 10 mg. were rubbed up with 1 ml. water and the insoluble residue was removed by centrifugation.

(*c*) The crystalline triosephosphate dehydrogenase was made by the method of Cori, Slein & Cori (1947). It was stored at 4° in 66% saturated $(NH_4)_2SO_4$ solution. Before use, it was centrifuged and the residue taken up in the desired medium.

(*d*) In the preparation of rat-muscle extract, used in measuring hexokinase activity, the cooled leg muscles were finely chopped with scissors, then ground in a mortar with 1·5 times the weight of ice-cold water. This procedure was carried out in a cold room. The brei was strained through fine handkerchief cotton and the filtrate used as quickly as possible.

Chemical preparations. The ATP was made by the method of Lohmann as described by Needham (1942) and the ADP by the method of Bailey (1949). The coenzyme I was made in some cases by the method of Williamson & Green (1940), in others by a modification of this method due to Ochoa (1948). The D-glyceraldehyde was kindly supplied by Mr B. C. Loughman, who prepared it by the method of Baer (Baer, Grosheintz & Fischer, 1939; Baer, 1945). We are indebted to Mr Loughman for the following characterization: the dimedon derivative (crystallizing in long prisms) had m.p. 196° and $[\alpha]_D^{18°} + 195°$ (in pure dry ethanol). The D-GA was stored as a semicrystalline syrup, slightly acid with acetic acid, at 4°; before use the pH of the solution was adjusted with 0·1 N-NaOH, using the glass electrode.

The glyceraldehydephosphate (GAP) was prepared by the method of Baer & Fischer (1943); much of it was kindly made for us by Mlle Bianca Tchoubar.

The phosphopyruvic acid was kindly supplied by Dr C. S. Hanes.

The sodium pyruvate was purified by the method of Robertson (1942).

Estimation methods. Glycolysis was followed by the method of Warburg. Lactic acid estimations were by the method of Friedemann, Cotonio & Schaffer (1927); glucose estimations by the method of Nelson (1944); phosphate estimations by the method of Fiske & Subbarow (1925); and fructose by the colorimetric method of Cole, Hanes, Jackson & Loughman (1951). Glyceraldehyde was estimated in the solutions by the method of Willstätter & Schudel, as described by Browne & Zerban (1941); when it had to be estimated in the presence of glucose, Rudney's (1949) modification of Nelson's method was used.

RESULTS

Effects of DL-*glyceraldehyde in experiments with muscle extract*

The system used was very similar to that described by Stickland (1941). The muscle extract (1·2 ml.) was made up to a final volume of 2·4 ml. This contained sodium phosphate, pH 7·6, 0·025 M; sodium bicarbonate, 0·26 g./100 ml.; glucose or glycogen, 0·32 g./100 ml.; ATP, 5×10^{-4} M; coenzyme I, 4×10^{-5} M; magnesium chloride, 4×10^{-3} M. Preliminary experiments were done with each new muscle extract to find the minimal amount of hexokinase which would give a steady glycolysis rate over 30–60 min.; 0·05–0·15 ml. of the solution described was usually needed. 95% nitrogen–5% carbon dioxide (v/v) was passed through the Warburg flasks and the experiments were carried out at 37°. Previous workers had shown that inhibition comes on slowly, and when GA was used, unless otherwise stated, it was added to the mixture immediately before the muscle extract; all vessels were then gassed for 5 min., and equilibrated in the bath for 10 min. before readings were begun.

Effect of glyceraldehyde on glucose ultilization. Fig. 1. (curves *A* and *B*) shows an experiment in which, with 0·0036 M-GA, inhibition of carbon dioxide production was practically complete from the beginning.

Estimations of glucose gave the following results:

	Glucose present (mg./sample)	
	At 0 min.	At 60 min.
Control	8·1	4·35
With 0·0036 M-GA	8·1	8·1

This shows that the inhibition under these conditions affects the first stage of glycolysis, the phosphorylation of glucose by hexokinase. However, the next experiment indicates, in confirmation of Stickland's results, that the inhibition is not directly upon hexokinase activity.

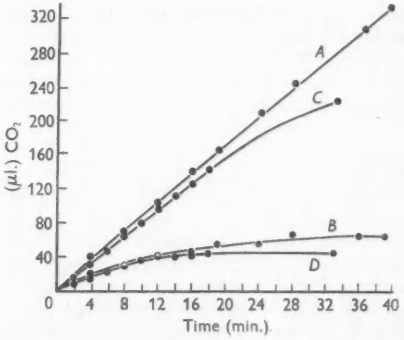

Fig. 1. Lactic acid formation from glucose in muscle extract + yeast hexokinase system. Curves *A* and *C* without GA; curve *B* with 0·0033 M-GA; curve *D* with 0·002 M-GA.

Curves *C* and *D*, in Fig. 1, shows the onset of inhibition in another extract when 0·002 M-GA was used. It will be seen that there is about 75% inhibition after 10 min. and that after 20 min. the inhibition is complete. Two extra manometers were set up, one with and one without GA, similar in all respects to those giving curves *C* and *D*, except that 0·06 M-fluoride was present and 0·004 M-ATP instead

of 0·0005 M was used; the required amount of ATP was placed in the side bulb. For initial examination of reducing power 0·5 ml. was removed from each vessel, and the manometers were gassed and equilibrated as usual. 10 min. after this the ATP was tipped in, and after another 10 min. period, samples again taken out. Half the usual amount of glucose was used in this experiment.

In this experiment no separate estimation was made of the GA present at the beginning and end of the experiment; but other experiments showed that with muscle extract the change in GA concentration is negligible; the change in reducing power may therefore be put down to utilization of glucose. It will be seen that under these conditions, where the ATP concentration is not limiting hexokinase activity, there is not more than 10 % inhibition.

	Glucose utilization (mg./sample) in the presence of GA, with high ATP concentration		
	Initial	Final	Used
Control	4·34	3·79	0·55
With 0·002 M-GA	5·25	4·75	0·50

Experiments were also done with the high ATP concentrations over a longer period (40 min.) and in absence of fluoride, lactic acid formation being measured chemically. The increased ATP lowered the inhibition by GA, but the effect was small, no doubt because the ATP is rapidly removed by ATPase:

	Lactic acid formation (mg./sample)	
	0·0005 M-ATP	0·002 M-ATP
Control	2·72	4·25
With 0·004 M-GA	0·55	1·64

The inhibitions were thus 80 and 61 % with 0·0005 and 0·002 M-ATP respectively.

Effect of iodoacetate. When the glycolytic system described was treated with a low concentration of iodoacetate (5×10^{-4} M) a state resembling that obtaining with GA could be produced, i.e. in which glucose breakdown was markedly inhibited, while glycogen breakdown was little affected. A typical experiment is shown in Fig. 2. The rate of glycolysis with glycogen was usually found to be less than the rate with glucose + hexokinase; it could be increased to some extent by addition of creatine (5 mg./sample). It might be expected that inhibitions in the slowly acting system might be less readily obtained, simply because the enzyme concerned did not so readily become the limiting factor; but, as the curves show, with the same extract the rate of glucose breakdown can be diminished by the iodoacetate far below that of glycogen breakdown, although the latter may start at a slower pace. Since TPD is the enzyme in the glycolytic series known to be most sensitive to iodoacetate, the effects described here, so similar to those obtained with GA, may be attributed to its partial inhibition.

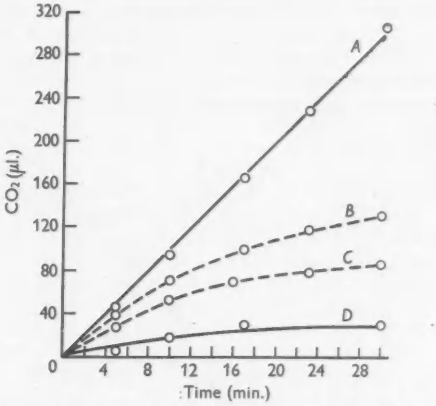

Fig. 2. Effect of 5×10^{-4} M-iodoacetate on lactic acid production from glucose and from glycogen in muscle extract + yeast hexokinase system. Curve *A*, glucose control; curve *B*, glycogen control; curve *C*, glycogen + iodoacetate; curve *D*, glucose + iodoacetate.

Effect of increased coenzyme I *concentration.* It was first observed by Mendel, Bauch & Strelitz (1931) that the inhibition of glycolysis by 0·001 M-GA in tumour slices can be markedly reduced by addition of traces of pyruvate and completely prevented by 0·001 M-pyruvate. Stickland (1941), using the muscle extract + hexokinase system with 0·003 M-GA, found the inhibition of 92 % reduced to 4 % by 0·0015 M-pyruvate, and he showed that the oxido-reduction

glyceraldehyde + pyruvate → glyceric acid + lactate

goes on so slowly at these low concentrations as to be quite insufficient as a means of removing the GA and thus reducing its toxic effect.

If the inhibition of glycolysis in this system is due to partial inhibition of TPD, the effect of the added pyruvate could be to increase the turnover at the TPD by providing for more rapid reoxidation of the reduced Co I. If this were the case, addition of Co I itself should have the same effect in alleviating the inhibition. As Fig. 3 shows, it was indeed found that increasing the Co I concentration about ten times (to $3{\cdot}3 \times 10^{-4}$ M) overcame the inhibition due to 0·004 M-GA. The increased Co I concentration has practically no effect on the rate of glycolysis in the control. This does not necessarily mean, however, that the TPD in the control is saturated with respect to Co I—we suggest that triosephosphate dehydrogenation is not the limiting reaction in the control, but has become so after the GA treatment. Anything

then enabling the partly inhibited enzyme to work with better efficiency is important in staving off the inhibition of glycolysis.

Effect of glyceraldehyde on enolase and pyruvic phosphokinase. Meyerhof & Wilson (1948) state that in one case at any rate (rat sarcoma) where traces of pyruvate greatly increase glycolysis in tissue extracts, this effect is due to a deficiency in enolase. The possibility was therefore considered that in the present experiments the striking effect of pyruvate

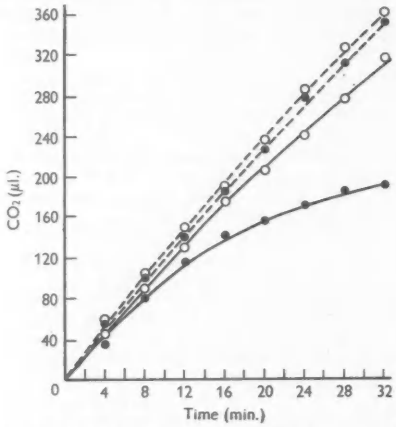

Fig. 3. Effect of increased Co I concentration upon lactic acid formation from glucose in muscle extract + yeast hexokinase in the presence and absence of 0·004 M-GA. ○-----○, no GA, $3·3 \times 10^{-4}$ M-CoI; ●-----●, no GA, 4×10^{-5} M-CoI; ○—○, 0·004 M-GA, $3·3 \times 10^{-4}$ M-CoI; ●—●, 0·004 M-GA, 4×10^{-5} M-CoI.

addition might depend on partial inhibition by the GA of enzymes responsible for converting phosphoglycerate into pyruvate. Two of these were tested, enolase and pyruvic phosphokinase, but no indication of inhibition was found.

The enolase used was a freeze-dried protein fraction kindly supplied by Miss A. B. Askonas who obtained it after precipitation of muscle extract between 0 and −5° with ethanol between 51 and 54% (v/v). A solution was made of 10 mg. in 2 ml. and centrifuged. The enzyme solution (0·1 ml.) was used in a final volume of 3 ml. with 0·07 M-phosphate, pH 6·7, 0·003 M-magnesium sulphate, 0·01 g./100 ml. glycine and 0·006 M-2-phosphoglycerate. Under these conditions the rate of reaction was found to be proportional to the enzyme concentration. The enzyme was mixed with the reagents except for the phosphoglycerate, and both control and sample + GA (0·0033 M) were allowed to stand 10 min. at 37°. The temperature was then adjusted to 20°, and the solutions were transferred to cells of the Beckman spectrophotometer; the reaction was started by adding the phosphoglycerate, and the rate of phosphopyruvate formation was followed by measuring the increase in optical density at 240 mμ. (Warburg & Christian, 1941). One of the experiments is shown in Fig. 4.

The source of the pyruvic phosphokinase was muscle powder made by precipitation of muscle extract with 3 vol. of acetone, filtering and drying rapidly. An extract was made with 25 mg./ml. water and centrifuged. Samples (0·5 ml.) of this extract were treated in the following way: to the control were added 0·1 ml. 0·2 M-phosphate buffer, pH 7·5 and 0·15 ml. water, while to the other were added buffer and 0·15 ml. 0·05 M-GA. The samples were then kept at 30° for 10 min.

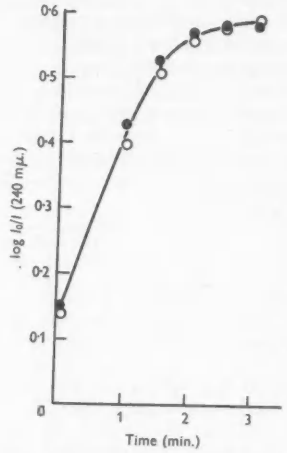

Fig. 4. Effect of GA (0·0033 M) on enolase: formation of phosphopyruvate from 2-phosphoglycerate. ○—○, no GA; ●—●, +GA.

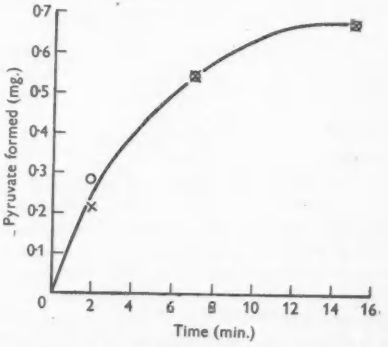

Fig. 5. Effect of GA (0·0066 M) on pyruvic phosphokinase: formation of pyruvate from phosphopyruvate. ○—○, no GA; ×——×, +GA.

The activities of these pretreated enzyme solutions were then tested in the following system: 0·3 ml. of each was used in a final volume of 1·6 ml. with 0·025 M-phosphopyruvate, 0·012 M-ADP, 0·02 M-phosphate, pH 7·5, 0·006 M-magnesium chloride, and 0·025 M-fluoride. The final concentration of GA with the GA-treated enzyme was 0·006 M. The experiment was carried out at 30°, and 0·3 ml. samples were removed at

intervals into 1 ml. 5% (w/v) trichloroacetic acid. The pyruvate formed was estimated by means of lactic dehydrogenase and reduced coenzyme I in the Beckman spectrophotometer, by measuring the decrease in density at 340 mμ. A final volume of 3 ml. was used, containing 0·1 ml. of the unknown solution, 1 mg. reduced coenzyme I, 0·05 mg. lactic dehydrogenase (a freeze-dried protein fraction made by Miss A. B. Askonas from muscle extract by precipitation with ethanol 23·5–31% (v/v) between 0 and −5°) and 0·03 M-phosphate buffer, pH 7·5. The results of a typical experiment are shown in Fig. 5.

Effect of added triosephosphate dehydrogenase. If the inhibition of lactic acid formation from glucose in the muscle extract + hexokinase system is due to partial inhibition of TPD, it might be expected that addition of extra TPD would delay the onset of the inhibition. This was indeed found to be the case.

TPD suspension of crystalline protein (0·5 ml.) in 66% saturated $NH_4(SO_4)$ was centrifuged at 20,000 rev./min. at 0°. The residue was dissolved in 0·6 ml. 0·03 M-pyrophosphate buffer, containing 0·0033 M-cysteine, pH 7·2; this solution was dialysed in cellophan tubing against 100 ml. of the same buffer at 0° for 1·5 hr. in an atmosphere of N_2. This TPD solution (0·3 ml.) was added to the usual system; 0·3 ml. of the pyrophosphate-cysteine buffer was added to the control.

One such experiment is illustrated in Fig. 6. GA was present from the beginning in two of the four Warburg vessels. A marked retardation by added

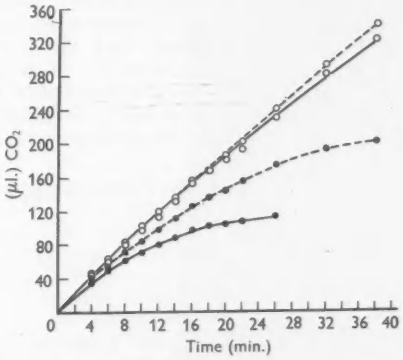

Fig. 6. Effect of added TPD upon lactic acid formation from glucose in muscle extract + hexokinase system in the presence and absence of 0·003 M-GA. ○—○, Control + 0·3 ml. pyrophosphate cysteine buffer; ○ - - - ○, control + 0·3 ml. TPD in pyrophosphate-cysteine buffer; ●—●, + GA + 0·3 ml. pyrophosphate-cysteine buffer; ● - - - ●, + GA + 0·3 ml. TPD in pyrophosphate-cysteine buffer.

TPD of the inhibition was observed; the addition of the extra enzyme made no difference to the rate of the control. About 0·5 mg. enzyme protein was added, an amount which probably did not increase the quantity of enzyme in the system by more than about 30–50%.

Experiments with crystalline triosephosphate dehydrogenase

In this part of the work the effect of the presence of DL-GA and of D-GA upon the rate of reduction of Co I by GAP in the presence of TPD was studied; the increase in reduced coenzyme concentration being measured by following the increase in density at 340 mμ. in the Beckman spectrophotometer. Conditions were adjusted to ensure that the rate of reaction was proportional to the amount of enzyme present. Arsenate was added to prevent accumulation of diphosphoglyceric acid and pyrophosphate buffer was used to minimize the effects upon the enzyme of any traces of heavy metals. The pH was 8·5, the optimal for TPD activity, unless otherwise stated. TPD is well known to be a sulphydryl enzyme; it is active without added cysteine, but in the presence of cysteine, the activity may be increased some fivefold. This increase in activity comes on gradually and is probably due to reduction of S—S linkages in the enzyme. On account of these progressive changes, and of the non-reproducible changes taking place in the enzyme solution on standing in absence of cysteine (see below), the observations were always made in groups of four Beckman cells. All four measurements were made within a few seconds of each other. Readings were taken at 0·5 min. intervals for 3 min., the rates of reaction at zero time calculated (cf. Rapkine, Shugar & Siminovitch, 1950) and the percentage inhibition calculated therefrom.

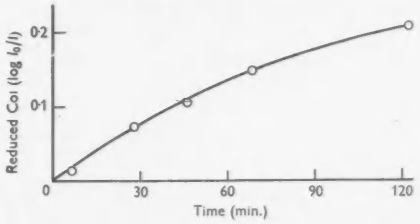

Fig. 7. Reduction of Co I by GA. Concentration of DL-GA, 0·0033 M. Concentration of Co I, 5×10^{-5} M. Temperature 25°, pH 8·5.

Non-enzymic reduction of coenzyme I by glyceraldehyde. It was observed in the course of this work that when Co I is allowed to stand with GA at pH 8·5 and room temperature, there is a gradual increase in absorption at 340 mμ. Fig. 7 shows the rate of the reaction with the usual concentration of Co I and 0·033 M-GA. At pH 7·0–7·5 the reaction is quite insignificant. Since most of the experiments described here were at pH 8·5, it is clear that a

correction is needed to allow for this non-enzymic reaction; the correction is small (about 0·01 in $\log I_0/I$) for the 3 min. experiments, but may become important in experiments of longer duration and with larger concentrations of either GA or Co I. A reaction of this sort between GAP and Co I was hardly detectable; on the other hand, reaction with benzaldehyde seems comparable in rate to that with GA.

Effect of DL-*glyceraldehyde upon oxidation of glyceraldehydephosphate by triosephosphate dehydrogenase in absence of cysteine.* The system used consisted of 10^{-4}M-GAP, 10^{-4}M-Co I, 6×10^{-3}M-arsenate, 0·033M-sodium pyrophosphate, pH 8·5, and 40–100 μg. TPD contained in 3 ml. The reaction was always started by addition of the GAP.

Fig. 8 and Table 1 illustrate the effect of the presence of 0·033–0·0033M-DL-GA in this system. It will be seen that the inhibition comes on at once, but even with the highest GA concentrations does not exceed 35–40 %. Attempts were made to increase this inhibition by allowing the samples to stand, with and without GA, for some minutes before addition of GAP. It was found, however, that quite apart from GA effects, the enzyme under these conditions at room temperature is not stable and its activity decreases or even occasionally increases. There were indications that the presence of GA sometimes afforded protection from the inactivation, and inhibitory effects of the GA might thus be masked. These attempts were therefore given up.

Some experiments of longer duration were done in which the GA was not added until the reaction in the presence of GAP was already in progress. That the inhibition comes on as well under these conditions is shown in curves *A* and *B* in Fig. 9. Curve *C* shows the increase in optical density in presence of only GA. If the correction due to the GA reaction, enzymic and non-enzymic, is taken into account, although it cannot be accurately assessed since the enzymic GA reaction is probably inhibited by the presence of GAP, the inhibition observed in curve *B* is even more marked and reaches approximately 25 % at the end of 12 min.

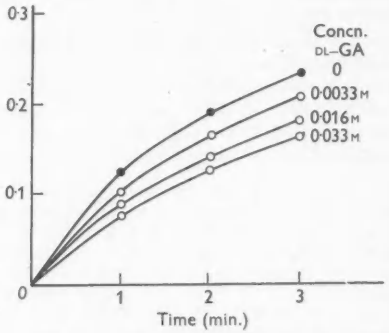

Fig. 8. Effect of various concentrations of DL-GA on oxidation of GAP by TPD. No cysteine present. Temp. 25°, pH 8·5.

Effects of DL-*glyceraldehyde upon oxidation of glyceraldehydephosphate by triosephosphate dehydrogenase in presence of cysteine.* In these experiments, 6×10^{-4}M-cysteine was present, and the system already described, with the exception of the co-enzyme and GAP, was allowed to stand until full activity was reached (10–20 min.). Only about 10 μg. of TPD were needed as the activity of the

Table 1. *Inhibition, by various concentrations of* DL-*glyceraldehyde, of the oxidation of glyceraldehyde-phosphate by triosephosphate dehydrogenase*

Exp. no.	Glyceraldehyde (M)	pH	Activities at zero time expressed in units of optical density: GAP alone	GAP plus GA	Inhibition (%)
1	0·033	8·5	0·170	0·116	34·0
2	0·033	8·5	0·382	0·246	35·6
	0·033	—	—	0·263	31·2
3	0·05	8·5	0·305	0·182	40·6
	0·033	—	—	0·213	30·0
	0·016	—	—	0·230	24·5
4	0·01	8·5	0·144	0·102	29·2
	0·0066	—	—	0·110	23·5
	0·0033	—	—	0·120	16·6
5	0·033	8·5	0·168	0·097	42·3
	0·016	—	—	0·115	31·5
	0·003	—	—	0·130	22·5
6	0·033	7·5	0·067	0·056	16·5
7	0·033	7·5	0·068	0·054	20·5

enzyme was so much greater. Coenzyme was then added to all the cells and the reaction started, with or without GA, by the addition of GAP. Under these conditions, very little, if any, inhibition was observed during the 3 min. of the test.

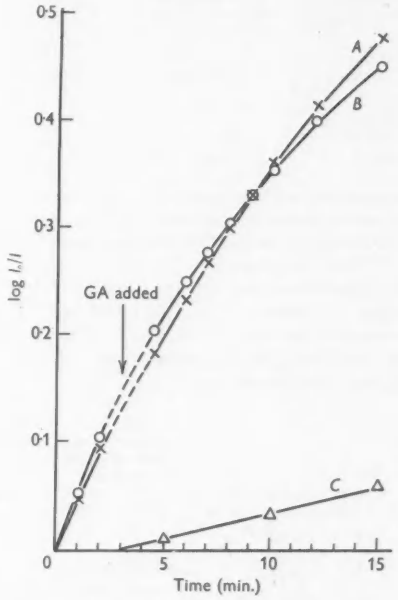

Fig. 9. Effect of addition of DL-GA (0·0033 M) after GAP oxidation has begun, pH 8·5, temp. 25. No cysteine present. *A*, GAP alone; *B*, GAP + GA; *C*, GA alone.

However, when the DL-GA was added at the same time as the cysteine, and the TPD plus GA plus cysteine were allowed to stand as before, large inhibitions were found. Fig. 10 shows one set of experiments where up to 75 % inhibition was obtained with 0·02 M-GA. Similar results were obtained when the enzyme was first activated with cysteine, and the GA then added and allowed to remain in contact with the enzyme for various lengths of time before the test. It was then seen that the inhibition was a function of time (Table 2).

It was further found that these large inhibitions, even when up to 80 %, could be reversed by subsequent addition of larger amounts of cysteine (final concentration 0·033 M).

Seven cells contained the usual reaction mixture, and TPD plus cysteine ($6{\cdot}6 \times 10^{-4}$ M in the cell) was added to all of them. After 30 min., the reaction in cell 1 was measured by addition of Co I and GAP as usual, and GA (0·0033 M) was added to cells 5–7. At 60 min., the reaction in cells 2 and 5 was measured and a concentrated solution of cysteine (0·033 M in the cell) was added to cells 4 and 7. After 15 min., the reaction in cells 3, 4, 6 and 7 was measured. The results of cells 1–3 (Table 3) show that in the absence of GA no appreciable change in activity in presence of cysteine occurred through the duration of the experiment. The activity in cell 4 indicates that these results represent maximum activities of TPD since an increased concentration of cysteine did not appreciably alter the rates obtained. The rate of

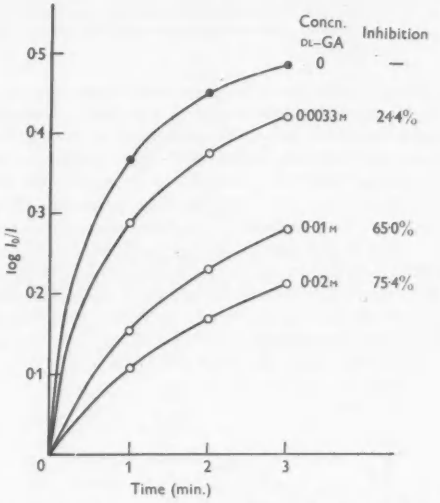

Fig. 10. Effect of DL-GA on oxidation of GAP by TPD, pH 8·5, temp. 25°, in presence of $6{\cdot}6 \times 10^{-5}$ M-cysteine. Cysteine and GA added 15 min. before measurement of activity.

Table 2. *Effect of time on the inhibition of triosephosphate dehydrogenase by* 0·007 M-DL-*glyceraldehyde in the presence of* 6×10^{-4} M-*cysteine*

(Cysteine added at time 0; GA added after 27 min.)

Time (min.)	Activities at zero time expressed in units of optical density: Without GA	With GA
34	0·362	0·287
43	—	0·214
53	—	0·042

reaction in cell 5 represents the decrease in activity in the presence of GA after 30 min. contact and in cell 6 after 45 min. contact. The increase in activity found in cell 7 clearly demonstrates that much of this loss can be recovered by addition of cysteine; in other words, that the inactivation is reversible. These results will be discussed more fully later in this section.

Table 3. *Reversibility of the glyceraldehyde inhibition of cysteine-activated triosephosphate dehydrogenase*

(System as usual + $6{\cdot}6 \times 10^{-4}$ M-cysteine; reaction started by the addition of CoI and glyceraldehydephosphate.)

Additions	Activities at zero time, expressed in units of optical density, when reaction was started at 30 min.	60 min.	75 min.
None	0·842	0·801	0·721
At 60 min. cysteine to final concentration of 0·033 M	—	—	0·716
At 30 min. glyceraldehyde to final concentration of 0·0033 M	—	0·305	0·028
At 30 min. glyceraldehyde to final concentration of 0·0033 M; at 60 min. cysteine to final concentration of 0·033 M	—	—	0·492

Since it is known that many aldehydes react reversibly with cysteine, combining probably through either the —SH or $—NH_2$ groups or both (see, for example, Schubert, 1935, 1936) the possibility was considered that, in presence of cysteine, the inhibitory effect of GA might be due to removal of free cysteine. This possibility is rendered unlikely by the fact that D-GA was found to have an inhibitory effect as great as twice the amount of DL-GA; it seems that the inhibitory effect is probably due entirely to the D isomer and it is unlikely that there would be any marked difference in rate of reaction of the two isomers with free cysteine.

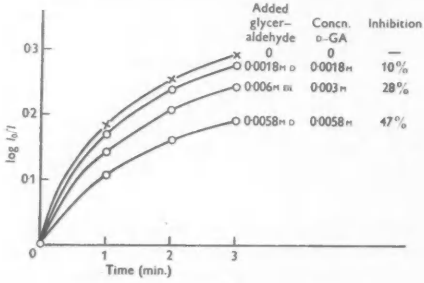

Fig. 11. Effect of D-GA and DL-GA on the oxidation of GAP by TPD; pH 8·5, temp. 25°. No cysteine present.

Experiments with D-*glyceraldehyde.* When this optical isomer became available studies were made to compare its effect quantitatively with that of DL-GA on GAP oxidation by TPD, both in the absence and presence of cysteine. Some of these results are shown in Tables 4 and 5 and in Figs. 11 and 12. Fig. 12 includes two sets of curves, one at pH 8·5, the other at pH 7·5. It is clear that the inhibition occurs also at the lower pH, and is indeed more marked.

This was the first time that an inhibitory effect of the D-form had been found. In the experiments of Needham & Lehmann (1937) on 4·5-day chick embryos, and of Mendel, Strelitz & Mundell (1938) on tumour slices, no inhibition was found with the D-form at concentrations where the DL-form was very effective; the inhibition in these cases was obviously due to the L-component.

An experiment was also done with D-GA in the absence of cysteine, to test the effect of GAP concentration upon the GA inhibition. Fig. 13 shows that when the GAP concentration was doubled, the inhibition was halved.

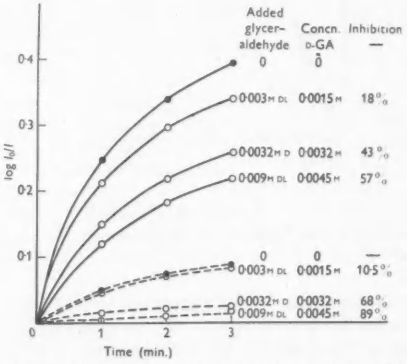

Fig. 12. Effect of D-GA and DL-GA on the oxidation of GAP by TPD, pH 8·5 and 7·5, temp. 25°, in presence of $6{\cdot}6 \times 10^{-4}$ M-cysteine. GA and cysteine added 15 min. before measurement of activity. ——, pH 8·5; - - - - - -, pH 7·5.

It is evident that a systematic kinetic study of TPD, with and without cysteine in the presence of the two substrates GAP and GA, would yield interesting results. The data so far collected in the present work are consonant with the following picture. In the absence of cysteine, reaction takes place at the active centres, D-GA acting as a competitive inhibitor with the GAP. When cysteine is present, a number of S—S groups are reduced and the activity of the enzyme is greatly increased. It is suggested that now the most marked effect of the GA is to put these —SH groups out of action by

Table 4. *Effect of various concentrations of* DL-*glyceraldehyde and* D-*glyceraldehyde on the activity of triosephosphate dehydrogenase*

Exp. no.	Form of glyceraldehyde concentration (M)	Concentration (M) as DL-glyceraldehyde	Activities at zero time expressed in terms of optical density: GAP alone	GAP plus GA	Inhibition (%)
1	D 0·014	0·014	0·125	0·080	36·6
	DL 0·016	0·008	—	0·114	9·1
	DL 0·033	0·016	—	0·103	17·5
2	D 0·0066	0·0066	0·142	0·108	24·0
	DL 0·0066	0·0033	—	0·125	12·0
	DL 0·0135	0·0067	—	0·117	18·0
3	DL 0·0033	0·0016	0·233	0·213	8·0
	D 0·0025	0·0025	—	0·203	13·0
	DL 0·01	0·005	—	0·160	32·0
4	DL 0·003	0·0015	0·278	0·285	0·0
	D 0·003	0·003	—	0·215	23·0
	DL 0·009	0·0045	—	0·208	27·0
5	D 0·0018	0·0018	0·267	0·242	10·0
	DL 0·006	0·003	—	0·194	28·0
	D 0·058	0·0058	—	0·141	47·0

Table 5. *Effect of various concentrations of* DL-*glyceraldehyde and* D-*glyceraldehyde in presence of* 6×10^{-4} M-*cysteine on the activity of triosephosphate dehydrogenase*

Exp. no.	Form of glyceraldehyde and concentration (M)	Concentration (M) as D-glyceraldehyde	Time of contact of GA and cysteine-activated TPD (min.)	Activities at zero time expressed in terms of optical density: GAP alone	GAP plus GA	Inhibition (%)
1	D 0·002	0·002	8	0·359	0·361	0
	DL 0·005	0·0025	—	—	0·343	5
	D 0·006	0·006	—	—	0·110	69
2	D 0·002	0·002	15	0·383	0·293	24
	DL 0·005	0·0025	—	—	0·183	52
	D 0·006	0·006	—	—	0·121	69
3	DL 0·0032	0·0016	15	0·254	0·202	18
	D 0·0032	0·0032	—	—	0·179	27
	DL 0·0092	0·0046	—	—	0·166	32
4	DL 0·0032	0·0016	15	0·365	0·300	18
	D 0·0032	0·0032	—	—	0·208	43
	DL 0·0092	0·0046	—	—	0·156	57

combining with them. This does not preclude concurrent competitive inhibition at the active centres. With the very high GA concentrations, the —SH groups produced by the cysteine are probably practically all obliterated and the activity falls to about that of GA inhibited cysteine-free enzyme.

There is obviously a great difference in behaviour of GA with and without cysteine; in the former case, inhibition appears at once but never goes very far, while in the latter case, inhibition comes on gradually but can reach 80–85%. This difference in behaviour might be tentatively correlated with competition of the GA with GAP in the cysteine-free enzyme, and predominant reaction of the GA with —SH groups in the cysteine-activated enzyme.

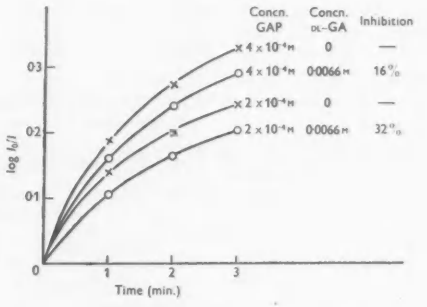

Fig. 13. Effect of GAP concentration on the GA inhibition of the oxidation of GAP by TPD; pH 8·5, temp. 25°. No cysteine present.

Experiments on the inhibition of hexokinase by glyceraldehyde

In the paper by Rudney (1949) experiments were carried out on extracts from brain, tumour and muscle, in the presence of a high concentration of ATP and of fluoride, to test the effect of GA on hexokinase. 0·0045–0·002 M-DL-glyceraldehyde gave 50–85% inhibition, varying somewhat with the different tissues. Yeast hexokinase was rather less sensitive, showing not more than 60% inhibition, even with 0·006 M-DL-glyceraldehyde. D-Glyceraldehyde tested on brain extract caused no inhibition, so that the effect must be due to the L-component.

An important difference between the tests used by Rudney and our own for the effect of GA on hexokinase activity lay in the glucose concentration. Rudney used 0·0025 M-glucose while we used 0·01 M. A series of experiments was therefore carried out with rat-muscle extract under conditions similar to those of Rudney, except that the glucose concentration was varied. The results are given in Table 6. GA was also estimated in all cases; the changes were very small and within experimental error, so that the change in reducing power was due to change in glucose content. The protective effect of increased glucose concentration on hexokinase activity in the presence of GA is clearly seen.

Some experiments were also carried out with fructose (Table 7); with this substrate, higher concentrations are needed to afford protection, which was only partial at 0·006 M with 0·002 M-GA. With the yeast hexokinase preparation of Meyerhof (1927), Stickland (1942) found no inhibition in presence of 0·02 M-fructose, with 0·02 M-GA. Lardy, Wiebelhaus & Mann (1950) find that L-sorbose-1-phosphate, at a concentration of only 0·0001 M, inhibits hexokinase activity in brain extracts. It is to be expected that this substance would be formed under the action of aldolase from dihydroxyacetone-phosphate and L-GA, and in inhibition following treatment with L-GA, the L-sorbose-1-phosphate may usually be the true toxic agent.

Effects of D-glyceraldehyde in experiments with muscle extract and hexokinase

In view of the proof that L-GA inhibits hexokinase while D-GA inhibits TPD it became necessary to test the effect of D-GA on the system used by

Table 6. *Effect of* DL-*glyceraldehyde upon the activity of muscle hexokinase in the presence of various concentrations of glucose*

(0·8 ml. Rat-muscle extract was used in a final volume of 2·4 ml. The other components were $NaHCO_3$, 0·26 g./100 ml.; $MgCl_2$, 0·003 M; ATP, 0·005 M; NaF, 0·08 M. The Warburg vessels were filled with 95% N_2, 5% CO_2 and were incubated at 30° for 12 min. 1 ml. samples were removed at the beginning and end of the experiment.)

Exp. no.	Glucose (M)	Glyceraldehyde (M)	Glucose disappearing: In control	Glucose disappearing: In sample and GA	Inhibition with GA (%)
1	0·006	0·003	0·537	0·528	None
	0·0025	0·003	0·508	0·129	75
2	0·0033	0·003	0·744	0·211	70
3	0·007	0·003	0·566	0·393	30
	0·005	0·003	0·566	0·278	50
	0·004	0·003	0·489	0·182	63
4	0·004	0·002	0·393	0·388	None
	0·0024	0·002	0·201	0·144	30
	0·0014	0·002	0·110	0	100

Table 7. *Effect of* DL-*glyceraldehyde upon the activity of muscle hexokinase in the presence of various concentrations of fructose*

(Components of the system as in Table 5, and experiments carried out in the same way, except that ATP concentration was 0·0066 M. GA, 0·002 M.)

Exp. no.	Fructose (M)	Fructose disappearing (mg.): In control	Fructose disappearing (mg.): In sample and GA	Inhibition with GA (%)
1	0·004	0·36	0	100
	0·0025	0·24	0	100
	0·0017	0·21	0	100
2	0·004	0·18	0	—
	0·005	0·18	0	—
	0·006	0·36	0·16	56

Stickland. It was found that D-GA does inhibit glycolysis in this system; its effect is rather greater than that of an equivalent amount of aldehyde in the racemic form, but not so great as the effect of twice the amount of racemic aldehyde. This indicates that the L-form is also having an effect, but to a less degree. It is doubtful whether, in the presence of the glucose concentrations used, this effect can be on hexokinase. Perhaps still another enzyme is affected by the L-isomer. Fig. 14 illustrates these experiments.

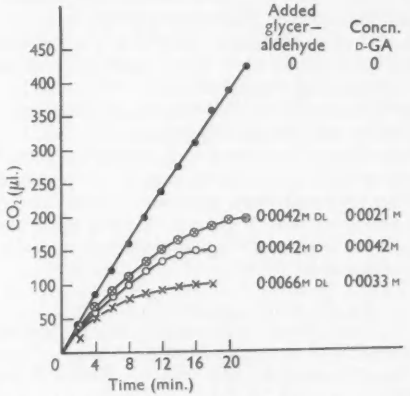

Fig. 14. Effect of D-GA and DL-GA on lactic acid formation in the muscle extract + hexokinase system.

An attempt was made to show the inhibition of TPD in muscle extract by spectrophotometric measurements. A 1 in 200 dilution of the extract (0·2 ml.) was used in the Beckman cells in place of the pure TPD with and without GA. Reduction of coenzyme proceeded with only a small inhibition for the first few minutes. Thereafter, although the density continued to increase slowly for a while with the GA-treated, a reversal took place in the control, the density becoming less. Clearly re-oxidation of reduced coenzyme was proceeding in the control but less so in the sample with GA-treated extract. This re-oxidation was unaffected by the presence of 0·016M-fluoride, as was shown in another experiment, and is probably to be put down to glycerophosphate dehydrogenase. It would thus appear that in the GA-treated extract, glycerophosphate dehydrogenase is inhibited. This circumstance makes it difficult to determine, by this method, whether TPD is also inhibited.

DISCUSSION

The results described may help to explain the fact that glycolysis in tissues has usually been found more sensitive to DL-glyceraldehyde than glycolysis in extracts. At the glucose concentrations generally used, the hexokinase of extracts, to judge by results with yeast and muscle extracts, would be protected; but with tissues, the glucose concentration at the enzyme surfaces within the cells may be much lower than that in the external medium. Further work is needed, however, to explain the mechanism of the glucose protection. Lardy *et al.* (1950) report that, with brain extract, increase in glucose concentration from 0·001 to 0·035M has no effect on the percentage inhibition of hexokinase by 8×10^{-5}M-sorbose-1-phosphate. Possibly when glucose prevents inhibition of hexokinase it does so, not by keeping a competitive inhibitor off the hexokinase, but by acting itself as a competitive inhibitor of aldolase, and thus slowing formation of the hexokinase inhibitor. Evidence for slight inhibition of aldolase by glucose and fructose has been given by Herbert, Gordon, Subrahmanyan & Green (1940) in the presence of 0·02M-fructosediphosphate; with minimal fructosediphosphate concentration, the inhibition might be much greater.

Inhibition of glycolysis by D-glyceraldehyde, via the partial inhibition of triosephosphate dehydrogenase, seems only to become important when there is high adenosinetriphosphatase activity in the system. Thus in the yeast hexokinase + muscle extract system, greater inhibition was found with D-glyceraldehyde than with an equivalent amount of DL-glyceraldehyde. In tissues, on the other hand, where adenosinetriphosphatase activity is localized and controlled, D-glyceraldehyde has comparatively little effect. It should be noted that both Needham & Lehmann (1937) and Mendel *et al.* (1938) observed some inhibition with D-glyceraldehyde; thus Needham & Lehmann (1937) found 25–30% inhibition with D-glyceraldehyde when DL-glyceraldehyde, at twice the concentration of total aldehyde, was giving about 85% inhibition. They explained the inhibition with D-glyceraldehyde as due to the presence of a small percentage of the L-isomer, since the optical rotation had fallen below that of the freshly prepared specimen. Baer & Fischer (1939) have since shown, however, that racemization cannot be the cause of the fall in optical activity, and there is, therefore, no reason to assume that the small inhibitions found are not due to D-glyceraldehyde itself.

The effect of pyruvate upon glycolysis in tissues inhibited by glyceraldehyde shows remarkable variation from one tissue to another and in the same tissue after different treatment. Thus striking alleviation of the inhibition is shown in the muscle extract-hexokinase system used by Stickland (1941) and in tumour slices (Mendel *et al.* 1931). But there is no effect on the inhibition in brain slices or extract (Baker, 1938). Rudney (1949) found no alleviation in brain, tumour and muscle extracts of the inhibition of hexokinase.

A possible mechanism for the pyruvate effect when D-glyceraldehyde is the inhibitor has been suggested earlier in this paper. Lardy *et al.* (1950) have suggested that when L-sorbose-1-phosphate is the inhibitor, pyruvate alleviation could be explained by supposing that increase in the ratio coenzyme I/reduced coenzyme I would favour oxidation of glyceraldehydephosphate rather than condensation with L-glyceraldehyde. Since both these suggested mechanisms depend on altering the balance in the activities of two enzyme systems, the variability of the response to pyruvate probably reflects only a variability in relative concentration of these enzymes in different tissues, or in their extractability when extracts are concerned.

SUMMARY

1. It is suggested that inhibition of triosephosphate dehydrogenase may play an important part in the well known inhibition of glycolysis by glyceraldehyde.

2. In a system consisting of muscle extract reinforced with yeast hexokinase, the inhibition can be delayed by addition of pure triosephosphate dehydrogenase. Other indirect evidence indicating the part played by inhibition of glyceraldehydephosphate oxidation in this system is also given.

3. The reduction of coenzyme I by glyceraldehydephosphate in presence of pure triosephosphate dehydrogenase is inhibited by DL-glyceraldehyde. This happens with the enzyme in absence of cysteine and also after cysteine activation. In the absence of cysteine, the degree of inhibition was found to depend upon the concentration of glyceraldehydephosphate. The inhibition of the cysteine-activated enzyme could be largely overcome by addition of more cysteine.

4. When D-glyceraldehyde was compared with DL-glyceraldehyde, it appeared that the inhibition of the pure enzyme was entirely due to the D-form. With the system consisting of muscle extract with yeast hexokinase, D-glyceraldehyde caused inhibition of glycolysis, but the effect was not as great as that of twice the amount of DL-glyceraldehyde.

5. In the latter system there was some evidence that glycerophosphate dehydrogenase was also inhibited by D-glyceraldehyde.

6. The inhibition of the hexokinase of rat-muscle extract by DL-glyceraldehyde was confirmed. It was found that the degree of inhibition depends on the concentration of glucose present; in the presence of 0·002M-glyceraldehyde the enzyme was entirely protected by 0·004M-glucose. About three times this concentration of fructose is necessary to protect the hexokinase.

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Antithyroid Substances

2. SOME MERCAPTOGLYOXALINES, MERCAPTOTHIAZOLES AND THIOHYDANTOINS

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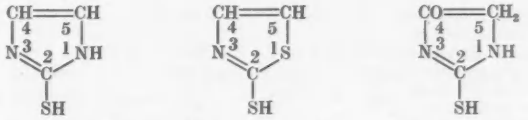
In a previous communication (Searle, Lawson & Hemmings, 1950) we reported on the antithyroid activity of a number of 2-mercaptoglyoxalines, the assays being based on the uptake of ^{131}I by the thyroid gland of rats after a single injection of the drug. We have extended this work to include a further series of 2-mercaptoglyoxalines, several 2-thiohydantoins and a number of 2-mercapto- and 2-amino-thiazole derivatives (Fig. 1). Many of these compounds were recently made accessible as a result of the work of Cook, Heilbron & collaborators (1947–9) and their structural relationship to compounds of known antithyroid activity seemed to us to warrant their investigation from this point of view.



2-Mercaptoglyoxaline 2-Mercaptothiazole 2-Thiohydantoin

Fig. 1.

We are also reporting on the effect of dietary administration over several weeks of some representative compounds of the different groups.

METHODS

The synthesis of new compounds reported on will be described elsewhere.

Assay technique. The screening test was carried out as already described (Searle *et al.* 1950). All drugs were administered by stomach tube, usually at a level of 0·05 mmol./kg. body weight. The dose of radioactive iodide (approx. 1 μc. in 0·5 ml. of 0·9% NaCl without added carrier) was injected intraperitoneally after 1 hr., and the rats killed after a further 4 hr. The ^{131}I uptakes of the thyroids of the dosed and control animals were then compared using a liquid counter, the results being expressed as the percentage depression of the mean ^{131}I uptake of the control animals. The results are summarized in Table 1.

In the longer-term feeding experiments the drugs were mixed at levels of 0·1 and 0·25% with the diet (either crushed cube diet or Glaxo powder diet, code no. RBSS-9). Each group of animals was matched for weight as in the screening tests, and each rat was weighed daily for some days before and during the experimental period. Finally, radioactive iodide was injected as described, and the ^{131}I content of the glands was determined after 24 hr. The results are shown in Table 2.

RESULTS AND DISCUSSION

Mercaptoglyoxalines

2-Mercapto-1-methylglyoxaline has already been reported by Stanley & Astwood (1949) as being 100 times more active than 2-thiouracil in man, and we have found this compound to be among the most active in rats. Jones, Kornfeld, McLaughlin & Anderson (1949) have reported on the antithyroid activity in rats of a number of 1-substituted 2-mercaptoglyoxalines, using the method of Astwood (1943) involving an estimation of compensatory thyroid hyperplasia for the assay. Of the compounds examined, the three most active were 2-mercaptoglyoxaline, and its 1-methyl and 1-ethyl derivatives, these being found to be only half as active as thiouracil. As reported in our previous communication (Searle *et al.* 1950) we found the activity of 2-mercaptoglyoxaline in rats to be about equal to that of thiouracil when tested by the radioactive iodine uptake method.

The only report of the clinical trial of a mercaptoglyoxaline is that of Reveno & Rosenbaum (1950) who successfully treated a small group of hyperthyroid patients with 2-mercapto-1-methylglyoxaline and found the activity of this substance to be about twenty-five times that of propylthiouracil. No toxic reactions were encountered at the dosage reported to be effective. The results of acute toxicity determinations in mice carried out with three representative mercaptoglyoxalines are shown in Table 3. Having in mind the activity of these compounds the figures would indicate that simple

Table 1. *Antithyroid activity of compounds as tested by the single-dose technique in rats*

Compound	Dose (mg./kg.)	No. of rats dosed	Depression of ^{131}I uptake (%)	Significance of differences from control*
2-Mercapto-1-methylglyoxaline	6	7	>93·2	*A*
1-Carbethoxy-2-mercapto-4-methylglyoxaline	9	14	63·4	*A*
2-Mercapto-4:5-diphenylglyoxaline	12·5	7	−2·7	*C*
4-Carbethoxyaminomethyl-2-mercaptoglyoxaline	10	7	−4·6	*C*
4-Carbethoxy-5-hydroxymethyl-2-mercaptoglyoxaline	10	7	16·2	*B*
Di-4:4′-(2-mercaptoglyoxalinyl)methane	10·5	7	−1·1	*C*
1-Carbethoxy-2-mercaptoglyoxaline	0·5	8	7·3	*C*
	2	8	58·8	*A*
	8·5	8	>94·9	*A*
	8·5	7	52·8†	*A*
4-Carbethoxyaminomethyl-1-carbethoxy-2-mercaptoglyoxaline‡	14	7	−48·5	*A*
Di-2:2′-(4-methylglyoxalinyl)disulphide	11	7	23·1	*B*
Di-2:2′-(1-carbethoxy-4-methylglyoxalinyl)disulphide	18·5	7	78·4	*A*
1-Benzoyl-2-thiohydantoin	25	5	93·7	*A*
3-Methyl-2-thiohydantoin	6·5	7	76·4	*A*
5-Methyl-2-thiohydantoin	6·5	9	71·2	*A*
2:4-Dithiohydantoin	7	5	21·1	*B*
5-*iso*Propylidene-2:4-dithiohydantoin	9	5	15·4	*C*
5-Acetamido-2-mercapto-4-*n*-propylthiazole	10	5	2·5	*C*
4-Ethoxymethylene-2-mercaptothiazol-5-one	9	5	−26·5	*B*
5-Amino-4-carbethoxy-2-mercaptothiazole	10	5	4·9	*C*
4-Carbethoxy-5-*cyclo*hexyl-2-mercaptothiazole	14	5	1·8	*C*
5-Acetamido-2-mercaptothiazole	9	5	2·8	*C*
5-Benzylideneamino-2-mercaptothiazole	9	7	−2·3	*C*
2-Mercapto-4:5-benzthiazole	8	5	−26·5	*C*
5-Amino-2-mercaptothiazole	7	5	−2·8	*C*
5-Amino-2-carbethoxythiothiazole	9·5	7	38·8	*A*
2-Mercaptothiazol-5-one	7	6	−33·9	*B*
2-Mercapto-4-*cyclo*pentylidenethiazol-5-one	10	5	25·1	*B*
5-Acetamido-2-carbethoxyaminothiazole	11·5	7	18·2	*C*
2-Benzamido-5-benzoylthioureido-4-phenylthiazole	21	4	29·5	*B*
5-Amino-4-carbethoxy-2-methylaminothiazole	10	5	0·3	*C*
2-Amino-4-carbethoxy-5-methylthiazole	9	5	0·4	*C*
2-Aminothiazole hydrogen maleate	11	6	9·2	*C*
5-Amino-2-benzamidothiazole	11	7	17·5	*C*
4:5:6:7-Tetrahydro-6-methyl-2-methylamino-7-keto-5-thionothiazolo (5:4-*d*)-pyrimidine	11	6	−3·4	*C*
2-Thiobarbituric acid	7	7	−13·5	*C*
Sulphanilylthiourea	12	7	−1·5	*C*
	50	7	14·0	*C*
Sulphanilamide	8·5	6	18·5	*C*
Sulphathiazole	13	7	25·4	*B*
Sulphadiazine	12·5	7	55·3	*A*
p-Hydroxybenzoic acid	100	8	0·8	*C*
Resorcinol	5·5	7	−0·9	*C*

* Probability: $P<0{\cdot}001$, *A*; $0{\cdot}1>P>0{\cdot}001$, *B*; $P>0{\cdot}1$, *C*.
† 24 hr. uptake experiment.
‡ Some decomposition in HCl.

members of the group are not unduly toxic. Comparable data for 2-thiouracil are also given in Table 3. Dieke, Allen & Richter (1947) report LD_{50} values of 1000 and 2000 mg./kg. for wild and domestic Norwegian rats respectively when 2-thiouracil was given orally.

Introduction of a phenyl group in the 4-position of the mercaptoglyoxaline nucleus gave a reduced antithyroid activity, and the 4:5-diphenyl compound, which had to be administered as a suspension in gum acacia solution owing to its insolubility, proved inactive. This latter finding is in agreement with results already published by Astwood, Bissell & Hughes (1945). The use of 1-carbethoxy-2-mercaptoglyoxaline, in which the imino hydrogen atom is replaced by an easily hydrolysable grouping, failed to prolong the antithyroid action of the glyoxaline as shown by a 24 hr. uptake test with this compound. Somewhat remarkable was the significant increase (48·5%) in radioactive iodine uptake produced by the *N*-carbethoxy derivative of 4-carbethoxyaminomethyl-2-mercaptoglyoxaline. Further investigation with this substance is proceeding. The high activity (78·4% depression) of the disulphide derived from 1-carbethoxy-2-mercapto-4-methylglyoxaline probably indicates a reductive

Table 2. *Effect of antithyroid substances on growth rate and thyroid activity of young rats*

Drug	Level in diet (%)	No. of rats	Duration of dosing (days)	Effect on growth	Thyroid wt. (mg./100 g.) Dosed	Thyroid wt. (mg./100 g.) Controls	Depression of 24 hr. ^{131}I uptake (%)
2-Mercaptoglyoxaline	0·25*	7	9	Immediate arrest of growth	28·9	12·9	>98·1
2-Mercaptoglyoxaline	0·1	7	29	Loss of weight after 9 days	53·2	9·5	97·0
2-Mercapto-4:5-dimethylglyoxaline	0·25*	7	9	Growth slightly reduced	26·8	12·9	43·0
1-Benzoyl-2-thiohydantoin	0·1	3†	29	Loss of weight after 11 days; fits after 15 days	39·2	9·5	—
2-Thiouracil	0·25*	7	12	Slightly reduced growth after 5 days	33·7	12·2	97·5
2-Thiouracil	0·1	8	32	Complete arrest of growth after 10 days	50·4	10·7	—
2-Mercapto-4:5-benzthiazole	0·25*	8	9	Growth slightly reduced	13·5	12·9	−2·7

* In these experiments the drugs were administered mixed with crushed commercial cube diet, this resulting in much less interference with growth than when the Glaxo diet RBSS-9 was employed. This supports the view (private communication from Dr J. Gross) that commercial diets prepared from fishmeal may contain appreciable amounts of thyroxine derived from the gastro-intestinal tract of the fish.

† These three animals died 4–5 days before the controls and other animals were killed. The three other animals in the group had been returned to normal diet (see text).

Table 3. *Acute toxicity of mercaptoglyoxalines in mice*

(LD_{50} values (mg./kg. body weight).)

Drug	Route of injection: Subcutaneous	Intraperitoneal	Intravenous
2-Mercaptoglyoxaline	350	640	1400
4-Ethyl-2-mercaptoglyoxaline	530	570	780
4-Carbethoxy-2-mercaptoglyoxaline	270	116	180
2-Thiouracil	4350	1160	825

fission in the organism to give the corresponding thiol known to be active, this change occurring less readily with the disulphide derived from 2-mercapto-4-methylglyoxaline.

The surprising inactivity of di-4:4′-(2-mercaptoglyoxalinyl)methane may be associated with its low solubility. It does, however, form a soluble sodium salt.

Mercaptothiazoles and aminothiazoles

No 2-mercapto- or 2-amino-thiazole tested showed activity comparable with the most active thiouracils and mercaptoglyoxalines, the largest depression of ^{131}I uptake (38·8 %) having been produced with 5-amino-2-carbethoxythiothiazole. Although many derivatives containing this ring system have been examined for antithyroid action, the only ones reported to show appreciable activity in rats are 4 - aminophenyl - 2′ - amino - 5′ - thiazolyl sulphide ('promizole sulphide') (McGinty & Bywater, 1945), the reduced thiazole, 2-mercaptothiazoline (Stanley & Astwood, 1947) and 3-(phenylaminomethyl)-thiazolidine-2-thione ('T.C. 105') (Rawson, McGinty, Peacock, Merrill, Wilson & Lockhart, 1948). In this connexion, the observation of Stanley & Astwood (1947) that 2-mercapto-4-methylthiazole is as active as thiouracil in humans but not in rats is of interest. Many of the derivatives of this class tested by us were rather unstable, and this may well have had some bearing on the low antithyroid activities observed. The effect of 2-mercapto-4:5-benzthiazole administered to young rats (50 g. wt.) in the diet is shown in Table 2.

Thiohydantoins

Jackman, Klenk, Fishburn, Tullar & Archer (1948) have already reported 5-methyl-2-thiohydantoin to have an antithyroid activity equal to that of thiouracil, but do not mention their method of assay. The three simple hydantoins tested by us had fairly high activity as is shown in Table 1.

The most active derivative reported here, 1-benzoyl-2-thiohydantoin (incorrectly reported as the 1-benzyl compound in Part 1, Searle *et al.* 1950) has also been administered mixed with the diet (Table 2). After 15 days at the level reported, the animals began to have intermittent convulsions soon after they were handled for purposes of weighing, etc. Three animals removed from the group after a further 8 days and given normal diet recovered quickly, but the remaining animals continued to lose weight and died 14 days later. The symptoms observed closely resembled the epileptiform fits described by Chick, El Sadr & Worden (1940) as being produced in rats after 4–5 months on a diet deficient in vitamin B_6. Examination of urine specimens from control and dosed animals for calcium and amino-acid levels showed no appreciable difference.

Miscellaneous compounds

The frequently reported goitrogenic activity of certain sulphonamides led us to examine three members of the group. The most active, sulphadiazine, has already been reported by Stanley & Astwood (1947) to be appreciably less active in humans than in rats. 2-Aminothiazole, active in humans, proved inactive in rats by our test. Our result for sulphanilylthiourea is in accordance with that reported by Astwood *et al.* (1945).

In our test, resorcinol showed no activity, although Doniach & Fraser (1950) found that this substance at the same dosage level, but using the subcutaneous route and shorter time intervals after administration, gave marked depressions of the ^{131}I uptake, in rats.

SUMMARY

A series of 2-mercaptoglyoxalines, 2-thiohydantoins, 2-mercapto- and 2-amino-thiazoles and some miscellaneous compounds has been tested for antithyroid activity in rats.

We wish to thank Glaxo Laboratories Research Division for their generosity in providing most of the rats used in this investigation and for determining the LD_{50} values. The radioactive iodide was obtained from the Atomic Energy Research Establishment (Isotope Division), Harwell. We are indebted to Mr R. A. F. Bullerwell and Dr A. H. Cook for providing some of the compounds used and to Mr A. W. Hemmings, Miss B. E. Petchey and Miss B. Lockyer for assistance during the course of the work. We wish to thank Prof. C. Rimington for his continued interest and advice, and the Rockefeller Bequest to University College Hospital Medical School and the Medical Research Council, for grants.

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